TOXICITY OF TOLYLTRIAZOLE TO GRAM-POSITIVE COCCUS MICROORGANISMS

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ABSTRACT (*Maximum 200 Words*)

Cold weather flight operations, military and civilian, require the use of de-icing fluids. Glycol, water, and additives (corrosion inhibitors and other performance enhancing chemicals) make up the de-icing fluids. Several of the additives are suspected toxins to microorganisms, therefore the EPA regulates the disposal of spent de-icing fluids.

Tolyltriazole, a corrosion inhibitor found in de-icing fluids, is a suspected human carcinogen. Tolyltriazole is known to be moderately toxic to microorganisms based on Microtox results. The purpose of this research was to determine whether tolyltriazole is toxic to isolated and/or consortia of common soil microorganisms' cultivated using propylene glycol and yeast extract. The microorganisms were exposed to increasing concentrations of tolyltriazole, with DO uptake rates being calculated. A decreased DO uptake rate serves as an indication of toxicity.

Culture 2 (Mars) had no adverse effects at any concentration, while culture 1 (Saturn) and culture 3 (*Bacillus*), showed inhibition at 1000 ppm. At lower concentrations (<500 ppm) no inhibition occurred in Saturn and *Bacillus*. At 500 ppm Saturn was inhibited, while *Bacillus* may have been stimulated. Microbial plate counts were prepared for diluted samples to determine impact on a microorganism's ability to replicate. Results indicate that replication was inhibited.

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Table of Contents

List of Figures

List of Tables

Abstract

Maintaining safe flight operations, military and civilian, in cold weather requires the use of de-icing fluids. Glycols, the primary component of de-icers, have been extensively studied. Water and additives such as corrosion inhibitors and other performance enhancing chemicals make up the remaining portion of de-icing fluids. Little research has been conducted on the additives, but several are suspected toxins to microorganisms, therefore the EPA regulates the disposal of spent de-icing fluids.

Tolyltriazole, a corrosion inhibitor found in de-icing fluids, is a suspected human carcinogen. It is also known to be moderately toxic to microorganisms based on Microtox results. The purpose of this research was to determine whether tolyltriazole is toxic to isolated and/or consortia of common soil microorganisms found in soil samples and cultivated using propylene glycol and yeast extract.

The microorganisms were exposed to increasing concentrations of tolyltriazole and the DO uptake rate was calculated. A decreased DO uptake rate serves as an indication of toxicity. Culture 2 (Mars) had no adverse effects at any concentration, while culture 1 (Saturn) and culture 3 (*Bacillus*), showed inhibition at 1000 ppm. At lower concentrations (<500 ppm) no inhibition was demonstrated in Saturn and *Bacillus*. At 500 ppm Saturn was inhibited, while *Bacillus* appears to have been stimulated. Microbial plate counts were prepared for diluted samples to determine impact to a microorganism's ability to replicate. The data indicates that replication was inhibited.

This work parallels that of Lt Chris Leonard and should be used in conjunction with his results to determine if any particular type of microorganism demonstrates a greater resilience or weakness towards tolyltriazole.

TOXICITY OF TOLYLTRIAZOLE TO GRAM-POSITIVE

COCCUS MICROORGANISMS

I. Introduction

1.1 Overview

Revised Federal Aviation Administration (FAA) requirements for de-icing have created a conflict between ensuring safe aircraft operations and protecting the environment. Passenger safety requires the use of Aircraft De-icing / Anti-icing Fluids (ADAFs) in the winter to remove and prevent the accumulation of snow, ice, and frost on aircraft. However, as the FAA increases de-icing operations, the Environmental Protection Agency (EPA) increases the requirements for storm-water runoff management (Mericas and Wagoner 38).

Two general types of ADAFs exist. The first is the Society of Automotive Engineers (SAE) Type I, consisting of glycol [propylene glycol (PG), ethylene glycol (EG), or diethylene glycol], water, coloring agents, and corrosion inhibitors [nitrates, silicates, etc.]. Type I ADAF is generally used to remove snow and / or ice adhering to the aircraft's surface (Bausmith and Neufield 459). The second general category is the SAE Type II ADAF, which is a Type I ADAF with the addition of synthetic polymers allowing it to adhere to the aircraft surface for longer periods of time. Type II is used to

prevent the formation of ice, snow, or frost on the aircraft prior to and during takeoff (Mericas and Wagoner 38).

Glycol based chemicals comprise approximately 50-80%, by weight, of the components found in ADAFs (Sills and Blakeslee 324, Hartwell *et al.* 1375). Additives, which make up 10-20%, include wetting agents, corrosion inhibitors, surfactants, thickeners, and other performance criteria agents (Hartwell *et al.* 1375). Water makes up the remaining portion of the ADAF (Cancilla *et al.* 1997:430). Studies have shown that formulated ethylene and propylene glycol de-icing and anti-icing mixtures are substantially more acutely and chronically toxic than pure propylene and ethylene glycol (Fisher and Knott 1108; Hartwell *et al.* 1383; Pillard 313). Pillard found that fathead minnows were more sensitive (relative to *C. dubia*) to the formulated deicers but less sensitive to the pure materials, which suggests that the mode of toxicity, and thus the toxicant, differed between formulated, and pure components (Pillard 314). Due to the lack of available information regarding the exact chemical formulation and concentration, relating specific environmental impacts to a particular chemical is difficult (Cancilla *et al.* 1997:430). The added toxicity may be attributable to all or some of the additives present in the ADAF mixture.

1.2 Purpose of Research

Previous theses efforts, Halterman-O'Malley (1997), Johnson (1997), Kellner (1999), and Burke (1999), studied various environmental impacts associated with ADAFs ranging from the biodegradation to the sorption of ADAFs to soil focusing on the biodegradation of ADAFs and the individual components in various soil types. Johnson

found that a high clay soil has higher oxygen consumption when exposed to mixtures of tolyltriazole and PG, indicating a greater degradation potential. Kellner and Burke focused on tolyltriazole and other additives used in ADAFs. Kellner discovered that, based on K_d values obtained through various experiments, tolyltriazole does not strongly sorb to soil (Kellner 5-3). Burke concluded that the rate of O_2 consumption in soil decreased with increasing concentrations of tolyltriazole (for constant PG concentration).

A high oxygen demand is associated with the biodegradation of glycol components (Pillard 314). PG is essentially non-toxic to humans and animals with a higher oxygen demand than EG. EG is more toxic to animals and humans (Sills and Blakeslee 326). Little information is available regarding the several additives found in ADAFs.

The chemical formulations for ADAFs are commonly proprietary (Cancilla *et al.* 1997:430). Cancilla identified many of the additives used in selected ADAFs, using a toxicity based bioassay analysis (1997:433). While the presence of additives can be identified using Cancilla's technique, it is difficult to know which chemicals each manufacturer uses and at what percentage of the total mixture that chemical is present. Cancilla confirmed the presence of benzotriazole and tolyltriazole in ADAFs (1997:433). Cornell has found that ADAFs used in North America typically contain between 1000-5000 ppm tolyltriazole for corrosion inhibition (1998:2). Tolyltriazole was shown to account for the majority of the Microtox activity in ADAFs (Cancilla *et al.* 1998:3834). Very little is known about the fate and effect of tolyltriazole, which has been shown to have high to moderate toxicity based on toxicological studies (Microtox) (Cancilla *et al.* 1998:3834).

Tolyltriazole has been detected in ground water near major airports increasing the need to learn more about the fate and effect of this chemical (Cancilla *et al.* 1998:3834). The moderate toxicity may negatively impact microbial populations found in the soil. Inhibition of the natural attenuation may result in accumulation of otherwise degradable products. The purpose of this research is to study the impact of tolyltriazole on soil microorganisms.

1.3 Research Objectives

1. Determine an effective methodology, based on dissolved oxygen (DO) concentrations, to measure the toxicity of tolyltriazole on microorganisms.
2. Evaluate the microbial toxicity of tolyltriazole on isolated soil microbial populations using DO consumption rates.
3. Compare results of microorganisms with different characteristics (shape and size) to each other to determine which microorganism characteristics appear to be most adaptable to tolyltriazole.

1.4 Scope

The research for this thesis effort was centered on the acute microbial toxicity of tolyltriazole to specific soil microorganisms upon initial exposure. Microorganisms were grown on a propylene glycol-rich environment, isolated, and cultivated on nutrient agar. The surviving microorganisms were identified, based on their Gram staining characteristic, positive or negative, and their shape, coccus (spherical) or bacillus (rod). DO readings were taken to determine the rate of DO uptake within a microcosm containing the isolated microorganisms and various concentrations of tolyltriazole. DO

readings were taken every nine minutes upon completion of the microcosm set-up. The rate of oxygen uptake was calculated using the recorded DO values. The oxygen uptake was, in turn, used as a measure of biological activity and therefore, indirectly as an indication of toxicity. Decreased microbial activity (lower DO uptake rate) served as an indication of toxicity. The DO readings from microcosms containing no tolyltriazole (control) and microcosms containing increasing amounts of tolyltriazole provided an indication of the toxicity of tolyltriazole.

Lt Chris Leonard (2000) conducted a complementary experiment on rod shaped microorganisms. The results from each effort can be compared to determine if one type of microorganism appears to be more capable of surviving in the presence of tolyltriazole.

1.5 Definitions

Biodegradation – The transformation to smaller molecules via oxidation and reduction mechanisms induced by the metabolic activity of native microorganisms that most organic substances undergo (LaGrega *et al.* 194).

Biological or Biochemical Oxygen Demand (BOD) – The measurement of dissolved oxygen used in the biochemical oxidation of organic matter (Tchobanoglous and Burton 71)

Microcosm – The standard test unit consisting of dilution water, inoculum, and toxicant solution (as required), contained within a 300 ml Wheaton BOD bottle (Marbas 3-1).

Octanol-Water Partitioning Coefficient (K_{ow}) – The concentration ratio of a compound, at

equilibrium, in octanol and water (Alexander 136).

Propylene Glycol – Non toxic chemical used in ADAFs as a freezing point depressant.

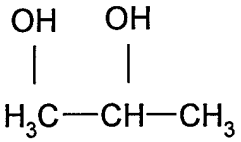
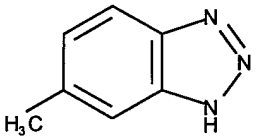


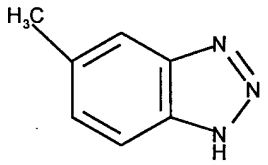
Figure 1. Molecular Structure of Propylene Glycol

Theoretical Oxygen Demand – A measure of oxygen required for the complete mineralization of an organic substance (Tchobanoglous and Burton 82).

Tolyltriazole – An additive used in ADAFs as a corrosion inhibitor. Low solubility in water (>0.1 g/100 mL @ 18 °C). Commercial tolyltriazole consists of two isomers, 40% 5-tolyltriazole and 60% 6-tolyltriazole (Chemfinder: Tolyltriazole; Cornell 8).



5-Tolyltriazole



6-Tolyltriazole

Figure 2. Molecular Structure of 5- and 6-Tolyltriazole

II. Literature Review

2.1 Background

Federal Aviation Regulations (FAR) prohibit aircraft takeoff when snow, ice, or frost has accumulated on wings, propellers, control surfaces, engine outlets, and other critical surfaces. The buildup of snow, ice, and frost on aircraft surfaces may significantly degrade the lifting properties and control of aircraft, possibly creating situations in which the passengers are put in danger (Cornell *et al.* 1). ADAFs are often used to prevent the build up of snow, ice, and frost, thus preventing dangerous situations.

ADAFs successfully remove and / or prevent the formation of snow, ice or frost because they depress the freezing point. Solutions of ethylene and propylene glycol can lower the freezing point of water to -13 and -59 °C respectively (O'Connor and Douglas 22). Freezing point depressants inhibit the development of a bond between the snow / ice and the surface to which the freezing point depressants are applied (Human Systems Center 80).

Two general classes of ADAFs exist, Society of Automotive Engineers (SAE) Type I and SAE Type II. Type I, a relatively thin liquid, is generally used as a de-icer. De-icing entails the removal of ice and snow from the aircraft's wings and fuselage in order to keep the aircraft clean until it is safely in the air. Type II, a more viscous fluid, is often used as an anti-icer because it clings to the aircraft surface for a longer period of time (Mericas and Wagoner 39). Anti-icing is often performed after de-icing to prevent further snow or ice accumulation during taxiing and takeoff.

2.1.1 Regulatory Background

Several changes regarding the use of ADAFs have occurred over the last couple of decades. Most significantly is the increased use of ADAFs in accordance with Code of Federal Regulations (CFR) Title 14, Part 121. In 1990 annual de-icing usage was estimated at 11.5 million gallons. Reports from airport operators indicate that usage has tripled since 1992 (Gallagher 1998:1).

ADAF discharge is monitored by a National Pollutant Discharge Elimination System (NPDES) permits, as mandated under The Clean Water Act originating from the Federal Water Pollution Control Act of 1972 (Bausmith and Neufield 459, Gallagher 1995:109). The FAA and EPA requirements concerning ADAFs are often in conflict; as the FAA requirements for de-icing and anti-icing increase, the EPA continues to increase the regulatory requirements associated with storm-water discharge (Mericas and Wagoner 39).

The EPA sets national effluent standards on an industry-by-industry basis, which is monitored with the NPDES permit program (Gallagher 1995:109). Prior to 1987 storm-water systems were not considered point sources, but in 1987 Congress established a schedule under which EPA was required to issue permits to regulate storm water discharges associated with industrial activities. The industrial activity's classification includes airports and all associated activities, thus requiring airports to maintain NPDES permits for storm-water discharge beginning in 1987 (Gallagher 1995:131).

2.1.2 Environmental Concerns

The primary environmental concern of ADAFs has been the high BOD associated with the degradation of glycol-based components. Originally glycols were assumed to be the primary source of ADAF toxicity (Pillard 311). While a high BOD is a significant environmental problem, ADAFs were otherwise assumed to be relatively benign because of the low toxicity exhibited by pure glycols. The acute and chronic aquatic toxicity of ethylene and propylene glycol is low. The acute and chronic oral toxicity to humans and terrestrial life is also low. None of the glycols in de-icer chemicals appear to be carcinogenic or mutagenic (Sills and Blakeslee 326).

Research conducted on pure glycol compounds and ADAF formulations indicate that the ADAF formulations have a greater toxicity than the pure glycol compounds. The greater toxicity is presumably due to the additives (Hartwell *et al.* 1383; Pillard 313). Table 1, adapted from Pillard, lists the results of acute toxicity tests using formulated ADAFs and pure ethylene and propylene glycol on different aquatic organisms. A lower LC_{50} indicates that the species is more sensitive to the toxicant. The results in Table 1 indicate that both species of invertebrates and fish are more sensitive to formulated ADAFs.

Table 1. Acute Toxicity Tests

Formulated ADAFs and Pure PG and EG

Species	Material[a]	Composition	48-h LC_{50}	96-h LC_{50}	NOAEC[b]
C. dubia	EG	Formulated	13140[c]		11610
C. dubia	PG	Formulated	1020		660
P. promelas	EG	Formulated	8540	8050	6090
P. promelas	PG	Formulated	790	710	600
C. dubia	EG	Pure	34440		24000
C. dubia	PG	Pure	18340		13020
P. promelas	EG	Pure	81950	72860	39140
P. promelas	PG	Pure	>62000	55770	52930

NOTES:
[a] EG = Ethylene Glycol; PG = Propylene Glycol
[b] No Observable Adverse Effect Concentration
[c] Concentrations are in mg/L

Common additives in formulated ADAFs include diethylene glycol, high-molecular-weight polymers, polyamines, triazoles, and urea. Other additives commonly used as corrosion inhibitors include sodium nitrite, sodium benzoate, borax, and benzotriazole (tolyltriazole) (Hartwell *et al.* 1384). Based on the toxicity results either one or more of the additives themselves are highly toxic or the additives have a toxic synergistic relationship with each other or the glycols (Hartwell *et al.* 1384).

2.2 Biodegradation

Biodegradation is an effective process in which microorganisms transform environmental contaminants. Contaminants may be transformed into more complex and possibly more toxic products or into simpler structures that may or may not be environmentally friendly. The most favorable outcome is the transformation of environmental contaminants into environmentally friendly compounds, such as water, carbon dioxide, and ammonia. Biodegradation will occur in nearly any medium as long

as water and microorganisms are available. The contaminant is often used as the energy or carbon source. Several factors act together in determining the rate and extent of degradation. The presence of toxins, possibly including the contaminant itself, will often impede the microbial activities of microorganisms.

Degradation rates are a function of microbial activity. Typically a higher microbial activity implies a higher degradation rate, as long as sufficient nutrients are available. As microorganisms become acclimated to their environment their populations may double and degrade the contaminant at a greater rate, provided nutrients are not limiting. Recording dissolved oxygen concentrations and calculating an oxygen consumption rate within a sample is used as a measure of microbial activity. Decreasing DO concentrations implies that the microorganisms are consuming oxygen and in turn degrading the contaminant. Oxygen consumption is measured as the slope of the curve produced from the DO readings over time where a steeper slope indicates a higher degradation rate. Simple straight-chained compounds (glycols) degrade more readily (have a steeper slope) than complex ring structured compounds (triazoles) (Alexander 150).

Efficiency of natural attenuation by microorganisms is subject to a variety of physical, chemical, and biological factors. Microbial populations function in a variety of environments (Alexander 196). The type of indigenous microbial population; temperature; pH; medium in which the degradation is occurring; availability of water, carbon source, nutrients, oxygen, and availability of electron acceptors all vary within each different environment. Aerobic degradation requires oxygen as the electron acceptor for use in the consumption of carbon sources. The primary benefit of

biodegradation is that it is a natural process, which converts contaminants into environmentally safe compounds.

Simple contaminants tend to degrade at faster rates than more complex structures. Glycols, straight-chained carbons with 2 hydroxyl groups, are easily degraded. Glycols tend to degrade into intermediates such as aldehydes and organic acids. These products are quickly degraded to the end products of carbon dioxide and water. More complex structures, such as cyclic or benzene rings, degrade at much slower rates than simple structures. Tolyltriazole contains a benzene ring with an attached methyl group and a nitrogen ring (see Figure 2) and does not degrade as easily or as quickly as the glycols due to its more complex structure.

2.2.1 Degradation of Propylene Glycol

Propylene glycol is a low molecular weight compound with a simple structure (see Figure 1). Microorganisms in water and soil readily degrade glycol under aerobic conditions. Raja used isolated strains of *Pseudomonas* and *Aerobacter* to determine the pathways of degradation. The PG was degraded to carboxylic and hydroxycarbonic acids by the *Pseudomonas*. Further decarboxylation to CO_2 was accomplished by the *Aerobacter* strains (Raja *et al.* 833-834). Final degradation for both species was observed to be CO_2 and H_2O (Raja *et al.* 830). The proposed degradation pathway, as suggested by Raja (838), is shown in Figure 3.

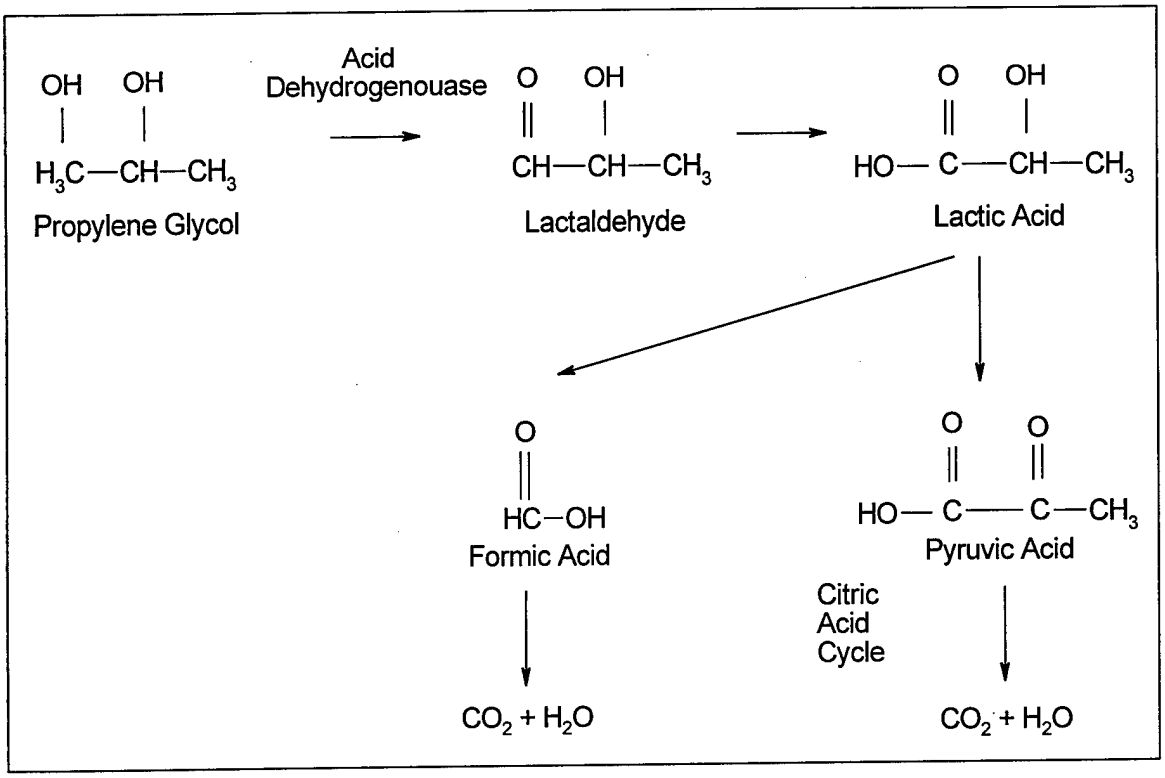


Figure 3. Proposed Biodegradation of Propylene Glycol

2.2.2 Degradation of Tolyltriazole

Typical ADAF formulations use a tolyltriazole mixture comprised of 40% 5-tolyltriazole and 60% 6- tolyltriazole by weight. According to literature some isomers appear to be more biodegradable than others (Cornell 1998:8). Cornell indicates that 6-tolyltriazole is biodegradable while 5-tolyltriazole is recalcitrant (not as easily degraded) (1998:8). A study performed by Cornell indicates that significant amounts of tolyltriazole will build up in the environment if only aerobic conditions exist. There is reason to believe that tolyltriazole may degrade under anaerobic conditions although no information is currently available. Cornell predicted the following anaerobic degradation pathway, Figure 4, for tolyltriazole (1998:12).

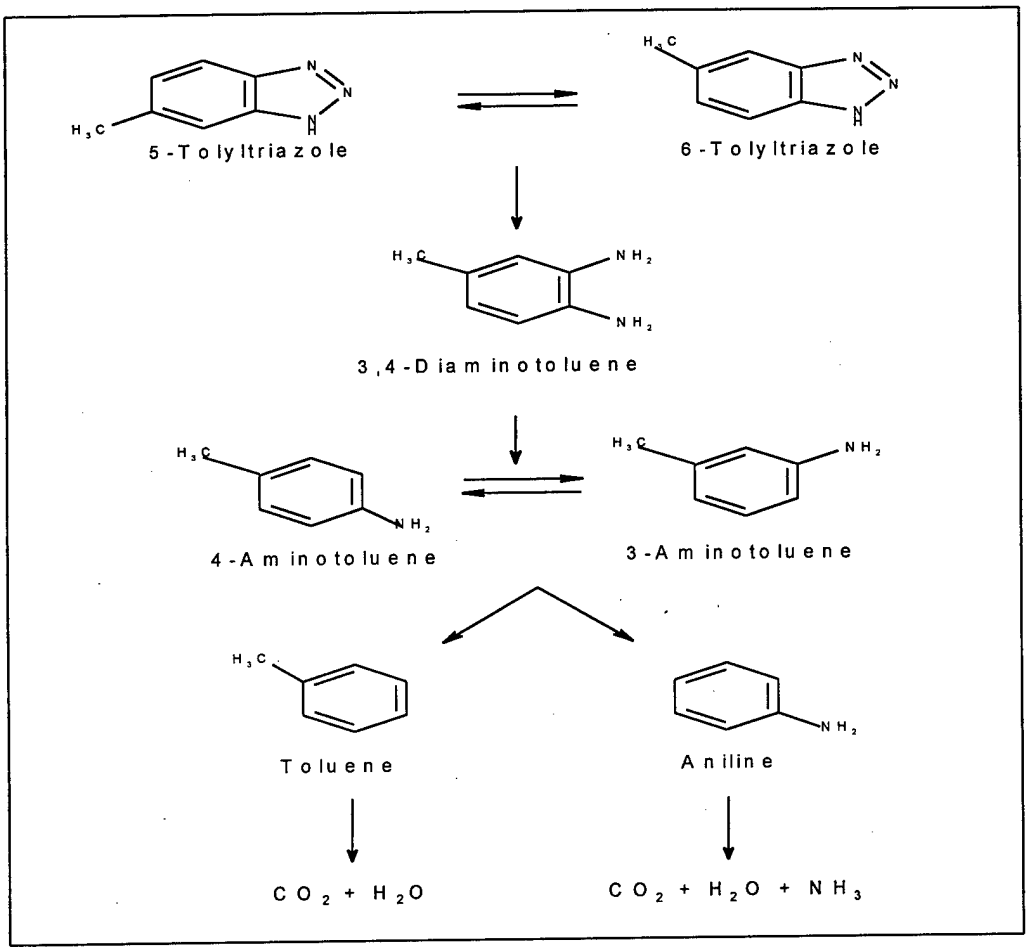


Figure 4. Proposed Biodegradation of Tolyltriazole

2.3 Toxicity

Toxicity is the measure of adverse effects manifested after uptake of a toxicant over a short period of time (acute) or a long period of time (chronic) (Hodgson *et al.* 10, 86). Toxicity values for various chemicals are commonly found by observing effects that materialize as the test subject is administered predetermined concentrations of the toxicant. Several variables factor in when performing toxicity tests on animals with even more issues factored in when addressing human toxicity. Human toxicity values are extrapolated from animal data. Little information is available for determining toxicity to

microorganisms. One common toxicity test for microorganisms, Microtox, has shown to be reliable and reproducible. Standard Methods has adopted a test, which is essentially identical to the Microtox test, Method 8050 B.

2.3.1 Microtox Toxicity Test

The Microtox test was developed in 1979 and is a rapid, low-cost, standardized, aquatic toxicity test (AZUR Environmental 1). The test utilizes a special freeze-drying technique providing shelf stable toxicity test organisms that can be reconstituted instantly and used when needed. The freeze-drying technology allows the production and distribution of standardized test organisms that are viable for up to 18 months. The test has achieved official standard status in several countries, including an ASTM Standard (D-5660) in the United States (AZUR Environmental 3).

The Microtox toxicity testing procedure utilizes luminescent bacteria, specifically the strain *Vibrio fischeri* NRRL B-11177, to measure the toxicity of environmental samples. When grown properly, the bacteria produce light as a by-product of cell respiration, which is fundamental to cellular metabolism and all associated life processes. Any inhibition to cellular activity (toxicity) will result in a decreased rate of cell respiration and a corresponding decreased rate of luminescence (AZUR Environmental 2). Greater toxicity results in a greater percentage drop in the rate of luminescence.

2.3.2 Dissolved Oxygen as a Measure of Toxicity

The measure of toxicity used for this thesis effort is the amount of dissolved oxygen (DO) consumed. Aerobic cell respiration, which is fundamental to cellular metabolism and associated life processes, requires the consumption of dissolved oxygen.

As long as microorganisms are performing life functions the rate of oxygen consumption should be high. The rate of oxygen consumption would be expected to decrease if a toxicant was present that prevented or inhibited normal cell respiration. In the presence of a toxicant, one would expect a decreased rate of oxygen consumption. Relative toxicity of different toxicant concentrations can be measured by comparing the change in oxygen consumption rates.

2.3.3 Plate Count as a Measure of Toxicity

A heterotrophic plate count is a procedure used for estimating the number of live heterotrophic microorganisms in water. Standard Methods (9215) discusses various procedures to perform plate counts. The pour plate method is simple and capable of handling volumes of sample or diluted sample. The colonies produced are smaller, more compact and show fewer tendencies to encroach on each other than surface growth plates such as spread plates. The number of colony forming units (CFUs) is an estimate of the number of live microorganisms (Standard Methods 9215).

For purposes of this thesis effort, pour plates were created for each microcosm. It was expected that, as the concentration of tolyltriazole increased, the number of CFUs would decrease. The decrease in the number of CFUs would indicate that tolyltriazole has an inhibitory effect on the microorganisms and is either toxic and killed microorganisms or prevented the microorganisms from replicating.

2.4 Theoretical Oxygen Demand

Theoretical Oxygen Demand (ThOD) is a calculation based on stoichiometry used to determine the amount of oxygen required to convert an organic carbon compound to

water, carbon dioxide, and ammonia. A higher ThOD indicates that the microorganisms require more oxygen to completely degrade the compound. Table 2 shows the calculations used to find the ThOD for propylene glycol and tolyltriazole.

Table 2. Theoretical Oxygen Demand for PG & Tolyltriazole

Basic ThOD Equation:

$$C_nH_aO_bN_c + (n + \frac{a}{4} - \frac{b}{2} - \frac{3}{4}c)O_2 \Rightarrow nCO_2 + (\frac{a}{2} - \frac{3}{2}c)H_2O + cNH_3$$

	Propylene Glycol	**Tolyltriazole**
Molecular Formula	$C_3H_8O_2$	$C_7H_7N_3$
Molecular Weight	$76.094 mgPG / mole$	$133 mgTolyltriazole / mole$
Stoichiometric Equation	$C_3H_8O_2 + 4O_2 \Rightarrow 3CO_2 + 4H_2O$	$C_7H_7N_3 + 6.5O_2 + H_2O \Rightarrow 7CO_2 + 3NH_3$
Molar Ratio	$O_2 : C_3H_8O_2 = 4.0$ $O_2 : CO_2 = 1.333$	$O_2 : C_7H_7N_3 = 6.5$ $O_2 : CO_2 = .9285$
ThOD	$= \frac{128 mgO_2}{76.094 mgPG}$ $= 1.68 mgO_2 / mgPG$	$= \frac{208 mgO_2}{133 mgTolyltriazole}$ $1.564 mgO_2 / mgTolyltriazole$

2.5 Glycol

Within the last five years there has been a shift from EG based ADAFs to PG based ADAFs. Ethylene glycol was the most popular de-icer, despite its toxicity and high biological oxygen demand (BOD). After a national shortage in 1994, airlines turned to PG as a substitute. From that point, EG never recaptured the market (Human Systems Center 76). Propylene glycol is not as toxic as ethylene glycol (Hartwell *et al.* 1375-1376).

Glycol based chemicals tend to degrade rapidly under aerobic conditions, preventing bioaccumulation, therefore; toxicity concerns with glycols are often transitory

due to the fast degradation (Hartwell *et al.* 1376, Pillard 314). The primary environmental concern with these chemicals is the high BOD. Airports often send their spent ADAFs to the local sewage treatment facility. The high BOD tends to disrupt the biological activities at the sewage treatment plant, incurring high costs to the airport (Cornell *et al.* 1998:1). The high BOD experienced in glycol receiving streams causes asphyxiation due to oxygen depletion, not direct toxicity (Pillard 312). One factor influencing the degradation rate is the chain length and molecular weight. Glycols are readily degraded in both soil and water environments.

Table 3. Chemical Characteristics of PG

Propylene Glycol Characteristics	Result	Reference
Boiling Point (°C) @ 760 mm Hg	188.2	Sax and Lewis (1989)
Freezing Point (°C) @ 760 mm Hg	-59	Sax and Lewis (1989)
Vapor Pressure (mm Hg) @ 20 °C	.08	Sax and Lewis (1989)
Solubility in Water	>10 g/100mL @ 21°C	Chemfinder(PG)

2.6 Tolyltriazole

Both Type I and Type II ADAFs contain rust and precious metal corrosion inhibitors. All rust and corrosion inhibitors are highly reactive and are designed to attach to and coat metal surfaces. By doing this, oxidation of the metal is minimized (Hartwell *et al.* 1375). Using a bioassay-directed isolation of ADAF fluid components, Cancilla and others identified the additive tolyltriazole (1997:430). Tolyltriazole is a common corrosion inhibitor used in virtually all anti-icing and engine coolant formulations as well as automobile antifreeze, cooling towers, and processes such as photographic development (Cancilla *et al.* 1998:3834). Cornell and others indicate that ADAFs are typically 0.5-0.6% (1000-5000 ppm) tolyltriazole (Cornell *et al.* 1998:2; Cornell 1998:2).

Other corrosion inhibitors include sodium nitrite, sodium benzoate, borax, and benzotriazole (Hartwell *et al.* 1384).

Tolyltriazole, a derivative of benzotriazole, is the common chemical name for 5(6)-methyl-1H-benzotriazole. The two isomers, 5 and 6 methyl-1H-benzotriazole, are both used when creating commercial grade tolyltriazole. Benzotriazoles and their derivatives, heterocyclic (aromatic) compounds, degrade differently than the glycol solvents or straight-chained alcohols, in which they are often dissolved (Human Systems Center 93).

Cornell and others found that, during Microtox experiments, the microorganisms were very sensitive to the addition of tolyltriazole (Cornell *et al.* 1998:10). At low environmental loadings (9 to 60 ppm), which modeled a dilute waste stream, the tolyltriazole conferred moderate toxicity to the *C. dubia*. However, at higher environmental loadings (> 600 ppm), representative of concentrated waste streams, the tolyltriazole conferred a very high toxicity to the *C. dubia* (Cornell 1998:8-9).

Cancilla and others found that the tolyltriazole exhibited significant toxicity using the Microtox test, as referenced in the table below (1997:433).

Table 4. Microtox EC_{50} Values for Benzotriazole[a]

Compound	Estimated Log(K_{ow})	EC_{50} (5 min) (mg/L)	EC_{50} (15 min) (mg/L)
1H-Benzotriazole[b]	1.27	41.13 ± 4.63	41.65 ± 11.01
5-Methyl-1H Benzotriazole[b]	2.16	5.69 ± 1.19	5.91 ± 1.11
5,6-Dimethyl-1H-Benzotriazole	3.05	0.72 ± 0.28	0.80 ± 0.33
Isolated fraction from Type I ADAF[c]	N/A	11.08 ± 0.49	12.39 ± 2.25

NOTES:
[a] Table from Cancilla *et al.* 1997:433
[b] Identified in Type I and Type II ADAF
[c] Isolated Benzotriazole

Limited acute toxicity data indicates that tolyltriazole is moderately toxic to *Lepomis macrochirus* and *Daphnia magna* (Cornell *et al.* 1998:2). Tolyltriazole is a weak organic acid and is relatively hydrophobic (Cornell *et al.* 1998:2). These properties indicate that tolyltriazole may accumulate in the subsurface or in sediments. The work of Burke (1999) suggests that the presence of tolyltriazole will reduce or slow the degradation of glycol or other degradable contaminants.

Some key properties of tolyltriazole are summarized in the table below. Of particular interest is the octanol / water partition coefficient. This coefficient serves as an indicator of the chemical's affinity for fatty tissues. The octanol / water partition coefficient is also useful in predicting the toxicity of a particular chemical. As a rule, organic solvents having a log K_{ow} ($K_{ow} \cong 0.6$) greater than 4.0 do not suppress microbial activity, whereas those with log K_{ow} ($K_{ow} \cong 0.3$) less than 2.0 are highly toxic (Alexander 137-138). This generalization does not apply to methylated benzotriazoles. Table 4, shown above, demonstrates that the toxicity increases with an increasing log K_{ow}. A regression analysis shows a significant linear relationship (r^2=0.999) indicating that partitioning into the microbe's bi-layer is an important aspect of the toxicity mechanism (Cornell 1998:2).

Table 5. Chemical Characteristics of Commercial Tolyltriazole

Tolyltriazole Characteristics	**Value**	**Reference**
Boiling Point (°C) @ 760 mm Hg	160	PMC Specialties (1996)
Melting Point (°C) @ 760 mm Hg	76-87	PMC Specialties (1996)
Vapor Pressure (mm Hg) @ 20 °C	0.03	PMC Specialties (1996)
Solubility in Water	<0.01 g/100mL @ 18°C	Chemfinder (Tolyltriazole)
Octanol/Water Partition Coefficient (log K_{ow})	2.16	Cornell (1998:7)

2.7 *Gram-Negative and Gram-Positive Characteristics*

One broad classification method for microorganisms is the process of Gram staining. Microorganism samples are stained, in the order listed, with the purple dye crystal violet, iodine solution, alcohol, and the red dye safranin. There are two classifications in which Gram stained microorganisms will fall; Gram-positive, microorganisms that retain the crystal violet dye and appear deep violet in color, or Gram-negative microorganisms, organisms which lose the crystal violet dye and appear red (Pelczar *et al.* 96). The reason microorganisms stain differently appears to be due to differences in the structure and thickness of the cell wall.

2.7.1 Gram-Positive Characteristics

Gram-positive cell membranes are simple in comparison to Gram-negative. Typically Gram-positive microorganisms have a cell wall that contains a large amount of peptidoglycan, a large polymer providing the rigid structure of the cell wall, making the cell wall appear thick. Peptidoglycan may account for 50 percent or more of the cell wall's dry weight (Pelczar *et al.* 122). Many Gram-positive microorganisms contain teichoic acids in their cell walls. Teichoic acids, polymers of glycerol and ribitol phosphates, attach to the peptidoglycan or to the cytoplasmic membrane. When the teichoic acids are negatively charged, they may aid in the transport of positive ions into and out of the cell (Pelczar *et al.* 122). Gram-positive cell walls contain none of the phospholipids that are common in the Gram-negative microorganisms.

2.7.2 Gram-Negative Characteristics

Gram-negative cells have an outer membrane covering a thin layer of peptidoglycan. The thin layer of peptidoglycan may only account for 10 percent of the cell wall's dry weight. The peptidoglycan layer is found in the periplasmic space, located between the cytoplasmic membrane and the outer membrane, a space that the Gram-positive microorganisms do not contain. The outer membrane serves as a selective barrier, controlling the flow of some substances into and out of the cell, just as the thick cell wall acts as a selective barrier in the Gram-positive microorganisms (Pelczar *et al.* 122).

The outer membrane is generally a barrier to large molecules, such as proteins, but is permeable to smaller molecules such as purines and pyrimadines, disaccharides, peptides, and amino acids. The outer layer is selectively permeable to molecules on the basis of their electric charge and molecular size.

2.7.3 Mechanism of the Gram Stain

Gram-positive microorganisms appear blue in color after Gram staining, while Gram-negative microorganisms appear red. During the Gram staining procedure, the microorganisms are treated with crystal violet (primary dye), then with iodine (mordant). This results in the formation of a crystal violet-iodine (CVI) complex within the cells. Upon washing the microorganism with ethanol, the lipid in the outer membrane of Gram-negative microorganisms dissolves and is removed. The outer membrane is disrupted, increasing the permeability (Pelczar *et al.* 124). The dye is washed away, decolorizing the Gram-negative microorganisms. The Gram-negative microorganisms are then stained

with the pink counter stain, safranin, and appear red in color. In Gram-positive microorganisms the ethanol shrinks the pores in the peptidoglycan, trapping the CVI dye complex causing the microorganisms to appear blue (Pelczar *et al.* 124).

III. Methodology

3.1 Overview of Experiment

This chapter describes the procedures and materials used in determining the microbial toxicity of tolyltriazole. Yellow Springs Instruments (YSI) 5905 and 5910 Biological Oxygen Demand (BOD) probes and YSI 58 and 5010 DO meters were used to collect DO concentrations within microcosms of isolated microorganisms exposed to increasing concentrations of tolyltriazole. The probes measure the concentration of DO within the microcosm, from which an oxygen consumption rate can be found. A decreasing rate of DO consumption over time indicates that the microorganisms may be sensitive to the test materials. Previous research conducted by Marbas (1996) used methods similar to this thesis effort.

The microcosms consisted of a measured amount of inoculate, a measured amount of dissolved tolyltriazole solution, and dilution water. Each experimental run differed in the inoculate solution being tested. Over the duration of the experiment, the DO values were recorded and increases or decreases in the rate of oxygen consumption were used as an indicator of microbial activity and, ultimately, toxicity.

3.2 Microorganisms

This section describes the process used to gather and cultivate the microorganisms used throughout this thesis effort.

3.2.1 Microorganism Source

For purposes of this research, microorganisms that thrive in conditions similar to those in an airfield were desired. Soil samples were taken from a clearing within a wooded area located near Building 470, Wright-Patterson Air Force Base, Ohio. The conditions in the field, few trees with patchy grass and weeds, were similar to those that might be found near an airfield. The top 10-20 centimeters of soil were removed and the sample was collected from the cleared area using a small shovel. The soil was placed into a plastic pail and transported into Building 470.

Ten grams of the soil sample and 100 ml of water were added to each of six 200 ml Erlenmeyer flasks. Increasing amounts of tolyltriazole (0.0 to 0.5 g) were added to the bottles to select microorganisms with some degree of tolerance. One bottle contained no tolyltriazole while the other five contained up to 0.5 g of tolyltriazole in 0.1 g increments. Each flask was stirred on a magnetic stirrer overnight.

3.2.2 Microorganism Isolation

STEP 1: The first attempt to grow microorganisms was accomplished using DIFCO noble agar. The agar solution was prepared by adding 2 ppm of PG, as the degradable carbon source; noble agar; and 2 300 mL HACH BOD nutrient buffer pillows (See table 6 for a breakdown of ingredients) as an inorganic nutrient source, to 500 ml of de-ionized water. The solution was autoclaved at 121 °C for 15 minutes in a Tuttnaur Brinkmann 3870 autoclave. Pour plates were prepared by adding 1.0 ml of the soil-tolyltriazole solution to the petri dish and then adding the agar solution upon cooling (~40°C). Three replicates were prepared for each soil-tolyltriazole solution along with two blanks

containing no soil-tolyltriazole solution. Upon solidification the pour plates were placed in a Cole-Parmer Ecotherm Chilling Incubator at a constant temperature of 28 °C.

Table 6. Hach BOD Buffer Pillow Components

Component	Percentage
Potassium Phosphate	< 5.0 %
Magnesium Sulfate	< 5.0 %
Calcium Chloride	< 5.0 %
Ferric Chloride	< 1.0 %
Other Components	< 1.0 %
Demineralized Water	To 100 %
NOTE: BOD Buffer MSDS (HACH Company)	

Growth was observed on the control plate and the plate containing 0.1 g/100mL tolyltriazole after 6 days. No growth was observed on the plates containing higher amounts of tolyltriazole.

STEP 2: The agar solution was prepared in the same way as Step 1 except 11.5 g of nutrient agar and 40 ppt of PG were added. After autoclaving and cooling the agar was poured into 20 petri dishes. The agar was left out overnight to solidify prior to placing the cultivated colonies from the Step 1 plates, onto the new plates.

Isolated colonies were removed from the control pour plates prepared in Step 1 and spread onto the new petri dishes using a sterilized platinum loop. The spread plates were placed in the incubator for three days.

STEP 3: A third agar solution was prepared in the same way as in Step 2 but contained 200 ppm of PG. The agar was poured in to the 20 petri dishes and allowed to solidify.

Colonies that appeared to be unique and uniform from the Step 2 plates were scraped using a sterilized platinum loop and placed onto separate new plates. Nine distinct microbial colonies were isolated.

STEP 4: Stock cultures were prepared in test tubes so that the nine colony types would be viable throughout the experiment. Nutrient agar supplemented with 200 ppm of PG was used as the growth medium for the microorganisms. The agar solution was poured into the test tubes and solidified at an angle creating a large surface area for growth. Once the test tube agar solidified each of the nine distinct organisms were transferred into individual test tubes using a sterilized platinum loop. Two replicate cultures were created for each microorganism. The test tubes were maintained in the incubator, allowing a stable growth environment.

3.2.3 Microorganism Classification

The microorganisms were classified in two manners, first by performing a Gram stain, and second by having the cultures identified by commercial laboratories.

3.2.3.1 Gram Stain Procedure

A Gram stain was performed on each of the nine microbial populations to further identify the microorganisms. A Fisher Diagnostics Gram staining kit was used and the associated procedure was followed. The results indicated that all of the isolated microbial populations were Gram-negative. The organisms were of different shapes and sizes, indicating that each test tube contained a different microbial colony.

The Gram staining was accomplished on microscope slides. The slides were then examined under a Zeiss microscope using 1000x magnification. The following table summarizes the shape of the nine microorganism samples.

Table 7. Gram-Negative Microorganism Shape and Size

Microorganism Sample	Shape
Mercury	Bacillus
Venus	Bacillus
Earth	Coccus
Mars	Coccus
Jupiter	Bacillus
Saturn	Coccus
Uranus	Bacillus / Coccus
Neptune	Coccus
Pluto	Coccus

3.2.3.2 Lab Analysis

Of the five coccus microbial populations, only two were chosen for use through out this thesis effort. After microscopic evaluation the chosen microbial populations were noticeably different in size from each other. Mars, Pluto, and Earth were all very small, and of the three, Mars (~0.5-1.5 μm) was chosen for further analysis. Saturn was definitely the largest (~0.5-2.0 μm) of the coccus microbial populations, and for that reason it was chosen. *Bacillus*, a Gram-positive microorganism, was chosen to use as a reference to compare microbial behavior among Gram-positive and Gram-negative microorganisms. 1Lt Chris Leonard (2000) evaluated the *Bacillus* culture and two other cultures that were rod-shaped, Jupiter and Venus.

Three commercial labs: Microcheck, INC., Midi Labs, and Microbial Insights each used different methods to evaluate the microorganisms. The methods used to identify the microorganisms included a fatty acid profile and a 16S rRNA gene sequence

similarity analysis. As indicated in the report (Appendix A) the microorganisms were different from the initial classification.

3.3 Dilution Solution / Inoculum / Toxicant Preparation

The following sub sections will explain the process of preparing the microorganism inoculum, the dilution solution used, and the toxicant solution.

3.3.1 Dilution Solution

The dilution solution was used to fill the remaining headspace in the BOD bottle after the tolyltriazole and inoculum had been added. The dilution solution consisted of de-ionized water supplemented with HACH BOD buffer. BOD buffer was added to ensure a consistent environment for the microorganisms, maintaining osmotic pressure similar to that the microorganisms were exposed to in the growth solution, and to ensure an adequate supply of inorganic nutrients. This solution was also used as a blank to ensure that outside contamination was minimized. The use of the blank microcosm will be explained later.

3.3.2 Microorganism Inoculum

The microorganisms were required to be in solution for the experiment. This was accomplished by creating a solution containing nutrients (organic and inorganic) in which the microorganisms would be able to thrive. One liter of de-ionized water was supplemented with 500 ppm PG, 500 ppm of yeast extract, and HACH BOD buffer (~ 4 300 ml pillows). The solution was autoclaved at 121 °C for 15 minutes to ensure sterility.

Four platinum loop swipes of the microorganism were removed from the agar plate and placed into two liters of solution. The solution was aerated, using a standard aquarium aerating stone, and continuously stirred overnight. The air was filtered through a 0.2 μL Millipore Millex®-FG_{50} filter to prevent contamination from microorganisms in the atmosphere.

3.3.3 Tolyltriazole Solution

Tolyltriazole is solid at room temperature and must be dissolved into solution for purposes of this experiment. Tolyltriazole is not highly soluble (<0.01 g/100mL @ 18°C). Based on the concentrations required for the experiment and the solubility limitations, a 5000 ppm stock solution was chosen. Five grams of tolyltriazole were added to 1 L of heated de-ionized water, the melting point of tolyltriazole is 76-87 °C. The solution was stirred until all solids were dissolved. The solution was cooled prior to usage in an experiment. The amount needed for a particular experiment, based on the concentration being tested, was calculated using the following equation.

$$(5000\,ppm)\left(\frac{NeededAmount(ml)}{300ml}\right) = DesiredConcentration(ppm)$$

The different concentrations used for the various experiments were calculated using the above equation and are shown in the table below.

Table 8. Tolyltriazole Solution Concentrations

Desired Concentration (ppm)	Required Amount of Stock Sol (ml)
10	.6
50	3
100	6
500	30
1000	60

3.4 Microcosm Setup

With the exception of the concentration of tolyltriazole and the type of microorganism added, all of the microcosms were prepared in the same manner. The microcosms used in this experiment were 300 ml biological oxygen demand (BOD) bottles.

3.4.1 Tolyltriazole Solution

The concentration of tolyltriazole used in the experiments was determined during trial experiments. A large range of values was used in order to capture the concentration at which adverse affects would be noticed. Experiments were performed using concentrations of 50, 100, 500, and 1000 ppm of tolyltriazole.

3.4.2 Dilution Water

BOD buffer was added to the de-ionized dilution water to ensure that the microorganisms were placed into a microcosm environment that had similar osmotic pressures and inorganic nutrients as found in the inoculum solution.

3.4.3 Inoculum Solution

The amount of inoculum used in the experiments was determined in trial runs. It was important to have a healthy population that would consume oxygen at a steady rate with a residual dissolved oxygen concentration of at least 2 mg/L at the end of the experiment. A 2 mg/L residual was chosen to ensure that the rate of DO consumption fell within the dynamic range. A series of trial experiments were performed with each microorganism to find the amount of inoculum that would meet the stated conditions. For the three microorganisms, the results of the trial experiments indicated that using 100 ml of inoculum met the specified conditions; greater than 1 mg/L DO consumption with a residual of at least 2 mg/L DO.

3.4.4 Microcosm Preparation

A microcosm was prepared by first adding the required amount of tolyltriazole solution. Second, 100 ml of dilution water was added to buffer the tolyltriazole solution prior to the addition of the microorganisms. Finally, 100 ml of inoculum was added and the remaining headspace was filled with dilution water.

Control microcosms were prepared in the same order as stated above, but no tolyltriazole solution was added. Three blank microcosms were prepared using only dilution water solution to ensure that cross contamination and outside contamination was not a factor in oxygen consumption. The blanks were also used to measure whether or not oxygen was being introduced into the system due to either poor seals or other avenues such as extended stopper removal.

3.5 Dissolved Oxygen Probe

The DO probes used for this thesis effort were YSI 5905 and YSI 5010 BOD probes in conjunction with the YSI 58 and YSI 5100 DO meters, respectively. The operating principals of the BOD probes are the same. The probes use a membrane-covered, Clark-type polarographic sensors with built in thermistors for temperature measurement. The electrolytic cell, consisting of a gold cathode and silver anode, is isolated from the environment by a thin permeable membrane stretched over the sensor. The membrane allows oxygen and other gases to enter, while preventing fouling of the cathode surface by impurities in the environment. Oxygen, upon entering the cell through the membrane, is reduced at an applied potential of -0.8 V referenced to the silver electrode. The partial pressure of oxygen in liquid (% air saturation) is directly proportional to the reduction current and to the concentration of dissolved oxygen (mg/L) at a particular temperature. The same partial pressure of oxygen will give different concentrations of DO depending on the temperature (YSI 5905/5010 BOD Probe Instruction Manual 2).

3.5.1 Probe Calibration

Manufacturer procedures were followed to ensure that the two YSI BOD probes were properly calibrated. Prior to every experiment, the probes were calibrated according to manufacturer's standards. In order to ensure the two probes produced the same result, comparison experiments were conducted after calibration as suggested by YSI procedures. First, the temperature readings needed to be stabilized to +/- 0.2 °C. Once this was accomplished, the YSI 5100 DO meter's probe was placed in a microcosm and

allowed to stabilize, then the percent DO was recorded. The second probe was then placed into the same microcosm, allowed to stabilize, and the percentage of DO was set to the same value as the YSI 5100 meter. The DO concentration (mg/L) values of each probe were compared to ensure the difference was less than +/- 0.2 mg/L. Once the stated conditions were met, the probes were each calibrated.

The probes were calibrated daily according to the procedures stated above. Upon calibration completion the probes were set in BOD bottles containing de-ionized water for 30 minutes to an hour allowing the probe to stabilize.

3.5.2 Consistency Experiment

A consistency experiment was performed to verify that the probes were able to detect DO concentrations within +/- 0.2 mg/L of each other in identical environments. An experiment was devised to ensure that the readings of each DO probe read DO concentrations within +/- 0.2 mg/L with in the same microcosm. Six BOD bottles were prepared each containing a different amount of sodium sulfite, Na_2SO_3. The solution was stirred until the Na_2SO_3 had dissolved. Sodium sulfite consumes DO and after a certain period of time the DO concentration will level off. This time period was determined in trial experiments. After 3 minutes the change in DO concentration was minimal, therefore it was determined that a time period of 5 minutes would allow adequate time for stabilization.

The first DO probe was placed in the bottle containing no Na_2SO_3 and allowed to equilibrate for 5 minutes, at which time the DO concentration was recorded. The DO probe was removed rinsed and placed in the next BOD bottle for 5 minutes. Once the

first DO probe was removed from the first BOD bottle, the second DO probe was placed in the first BOD bottle. The second DO probe was allowed to equilibrate for 5 minutes before the DO concentration was recorded. The experiment was performed to determine the difference in each DO probe. The DO probes were found to be comparable. The average difference between DO concentration readings of the two probes, 0.055 mg/L, was less than that allowed by the manufacturer's guidelines (0.2 mg/L). Reference Appendix C for test results.

3.6 Experimental Setup

Eighteen microcosms were prepared for each experimental run. Microcosms consisted of blanks (de-ionized water), controls (0 ppm tolyltriazole), and four different tolyltriazole concentrations, with three replicates prepared for each. Each probe was used to record the oxygen values of nine microcosms.

The microcosms were prepared on a staggered time schedule to ensure that all microcosms were freshly prepared prior to the initial DO reading. The BOD probe was rinsed with de-ionized water prior to all DO measurements. The probe was inserted into the BOD bottle firmly to ensure an airtight seal and the microcosm was continuously stirred. The probe was allowed to stir in the bottle for one minute prior to recording the DO measurement. Eight cycles were performed to ensure that an adequate number of points existed for analysis of the DO rates. Between readings a glass stopper was placed in the BOD bottles to prevent the introduction of outside oxygen by providing an airtight seal.

3.7 Dry-Weight Procedure

Dry weight was calculated as an indirect measure of microbial mass. Dry weight was also used as a check to ensure that the inoculum solution was well mixed. Three replicates were taken and the average weight of the three calculated.

Whatman glass microfibre (1 μm pore size) filters were rinsed with de-ionized water, placed into an aluminum weigh boat and dried in a Blue General Signal Co. muffle furnace at 600 °C for 3 hours. The aluminum weigh boats were removed from the muffle furnace and placed in a Whatman desiccator for storage until used. The tarred initial dry weight of the filters was recorded, just prior to filtration, using a Mettler Toledo AB204-5 balance. The filter was placed on a vacuum filter and a 20 ml sample was filtered through before experimental set-up. This was repeated three times. After filtering the inoculum contents the filters were placed in their respective aluminum weigh boats and placed in the Precision Scientific Thelco laboratory oven at 104 °C for 6 hours. The final dry weight was recorded.

3.8 Colony Count

The ability of the microorganisms to replicate was measured using a colony count. Upon completion of the experiment, pour plates were prepared in accordance with Standard Methods 9215, for each microcosm. The samples from each microcosm were diluted and then 500 μl of the diluted solution was placed into the petri dishes.

Test tubes containing 10 ml of de-ionized water, supplemented with BOD buffer, were prepared. Twenty micro liters were removed from each microcosm and pipetted into the test tubes. Each test tube was shaken and 500 μl from the diluted sample was

placed into petri dishes. Pour plates were prepared using nutrient agar. Upon solidification the plates were placed into an incubator for 24 hours.

Plate counts were performed using a Leica Quebec Darkfield Colony Counter. In the case that there were too many colonies to count, one grid was counted and multiplied by the number of grids. In some situations there were too many CFUs too numerous to count, even using the grid method, and a number could not be recorded.

3.9 Statistical Analysis

The first objective of this thesis effort was to determine if tolyltriazole was toxic, to the isolated microorganisms' at the tested concentrations. Average DO consumption rates for unexposed microorganisms were compared to the average DO consumption rates of microorganisms exposed to different levels of tolyltriazole. Several statistical tests exist that can be used to analyze the data generated through out this thesis effort.

An Analysis of Variance (ANOVA) was conducted to determine if a significant difference in the means existed. The ANOVA test is used for multiple comparisons of means within an experiment with two or more treatments from two or more numerical populations (Devore 390). For this thesis, it is assumed that the experimental means will be statistically different from the control means if tolyltriazole is toxic to the microorganisms. An ANOVA allows one to quickly assess whether further statistical analysis is required. Based on ANOVA results additional statistical analysis was required.

3.9.1 Dunnett's Multiple Comparisons of Means

The Dunnett's comparison is a statistical test used when comparing several experimental means against that of a control. The Dunnett's comparison, unlike multiple t-tests, allows one to control the alpha value (rejecting the null hypothesis when it is true). As the number of comparisons increases in a t-test the alpha value increases. However, a Dunnett's comparison only does comparisons of each experiment mean to that of a specified control, maintaining a specified alpha value. The Dunnett's comparison analysis was performed by the statistical software package JMP® Start Statistics (Sall and Lehman, 1996).

The familywise Type I error will not exceed 0.05 (the specified per comparison Type I error rate) in a Dunnett comparison, while in other comparison calculations the familywise Type I error will exceed the per comparison Type I error (Sheskin 340). The equation used to calculate the familywise Type I error rate is shown below (Sheskin 340):

$$\alpha_{fw} = 1 - (1 - \alpha_{pc})^c$$

For the conditions in this thesis, five comparisons, the familywise Type I error rate was found to be 0.226, much greater than the per comparison Type I error of 0.05. The Dunnett's comparison proved to be the most useful, due to the fact that it was able to keep the Type I error rate low by adjusting the level of significance for each comparison.

Each microorganism's control DO uptake rate (identified as the control) was compared to the uptake rates at 50, 100, 500, and 1000 ppm tolyltriazole (identified as the experimental). The Dunnett's comparison identified any concentrations that produced

statistically significant differences from the control at a familywise 95% level of confidence. See Appendix B for detailed Dunnett's test procedures.

3.9.2 Two-Tailed t-Test

The two-tailed two independent sample t-test was chosen to determine if high concentrations of tolyltriazole affected the DO probe membrane. The two-tailed t-test performs a comparison between two means. The null hypothesis states that the two means being compared are the same. A two-tailed test was chosen because the null would only be rejected if a large enough difference existed between the two means (positive or negative). The goal of the experiment was to determine whether or not the probe membrane was significantly influenced by high concentrations of tolyltriazole.

3.10 Early Experiments

Determining the final methodology described in the preceding section was an iterative process. Several experiments were run, problems identified and corrective measures taken. Some corrective measures proved to be useful while others made no difference in the results.

3.10.1 Propylene Glycol Stock Solution

The PG stock solution is the growth medium in which the microorganisms were placed once they had been scraped from the petri dish. Original experiments used a prepared stock solution (1 L) containing 500 ppm of PG and BOD buffer. During the early experiments the activity of the microorganisms, as measured by DO uptake, was quite low. An additional food source, 500 ppm of yeast extract, was added to ensure

there was an adequate food source. The activity of the microorganisms improved and this change was incorporated into the final methodology.

Originally the solution was stirred overnight to allow the microorganisms to replicate. When the experiment began the initial DO concentration was often around 4 mg/L. This value was low and often went to zero before the experiment was over. The problem was resolved by aerating the microorganisms overnight. A standard fish aquarium aerator was used and between uses it was disinfected with hydrogen peroxide and thoroughly rinsed with de-ionized water.

3.10.2 Dilution Water

The dilution water was used to fill the remainder of the microcosm after the tolyltriazole and microorganism solutions had been added. During early experiments only de-ionized water was used. After examining some of the procedures used, it was decided that BOD buffer should be added to the dilution water. The microorganisms were being cultivated in a solution containing inorganics and then transferred to a solution containing no inorganics. In order to maintain a stable environment, BOD buffer was added to the dilution water.

3.10.3 Microcosm Stirring

During the first experiment the microcosms were not stirred. It was assumed that the stirring of the DO probe would be sufficient. Soon after it was decided that the DO probe stirring was not enough. Magnetic stir bars were placed in each microcosm and the microcosm was stirred while the readings were taken. This small procedure change resulted in more consistent results between microcosms of similar composition.

3.10.4 Microcosom Preparation

Several different variations were tried when determining the microcosm preparation that resulted in the most consistent results. The first issue to be addressed was the volume of microbial solution to be added to the microcosm. The amount of microbial solution added to the microcosm needed to result in a noticeable DO decrease, but did not cause the DO to reach zero during the duration of the experiment. Initial experiments used 10 and then 50 mL of microorganisms, but the decrease in DO was not noticeable for all of the microorganisms. Finally, 100 mL was tried and the results were consistent for the different microorganisms. Some dropped at a greater rate, but none of them dropped to a level that forced the experiment to be stopped.

During initial experiments the four concentrations of tolyltriazole that were tested were 10, 50, 100, and 500 ppm. It was determined that the 10 ppm microcosm did not have any noticeable impact on the microorganisms. In order to get a better range of possible toxicity; the 10 ppm microcosm was replaced with a 1000 ppm microcosm. This finding was incorporated into the final methodology.

The last major alteration was the order in which the components were placed into the microcosm. Originally the required amount of the concentrated tolyltriazole solution was added, then the required amount of microorganism solution was added, and finally the remainder of the bottle was filled with dilution water. After trying to determine why the results were not consistent, the fact that the microorganisms were momentarily exposed to a concentrated tolyltriazole solution (5000 ppm) came to light. The short-term exposure to the high concentration may have inhibited or killed the microorganisms. This concern was resolved by adding the tolyltriazole solution, 100 mL of dilution water,

100 mL of microorganism solution, and the remainder of the bottle was filled with dilution water.

3.10.5 DO Probe Cleaning

The most significant change that resulted in more consistent results was the cleaning of the probes. The probes' membranes were replaced every week. However, a complete cleaning of the anode was not accomplished. After troubleshooting all possible reasons for the inconsistent probe readings, it was determined that the anode needed to be thoroughly cleaned. After the cleaning was accomplished, according to the user manual, the readings were more consistent.

IV. Data Analysis

4.1 Overview of Data Analysis

Visual and statistical analysis were conducted on both the DO uptake rate data and the colony plate count data. Visually one could determine differences between the different concentrations of tolyltriazole. The DO uptake rate graphs were examined and, in general, lower uptake rates were observed for microcosms containing greater concentrations of tolyltriazole. Upon examination of the petri dishes after a 24-hour period, one could see a difference in the number of colonies present between the lower and higher concentration microcosms.

4.2 Equipment Standardization

Comparison of the two DO probes was conducted to ensure that the results from the two probes were consistent, as specified by the manufacturer's guidelines. The procedures outlined in the methodology were followed with the goal of showing reproducibility of the probes' readings.

4.2.1 Consistency Experiment

An experiment was performed to determine the capability of the probes to detect the same DO concentration in different microcosms. Different amounts of sodium sulfite, Na_2SO_3, were used to consume different amounts of DO within each BOD bottle. At higher concentrations of Na_2SO_3, the concentration of DO was lower. The experiment determined that the probes were precise (degree of agreement between replicate

measurements of the same quantity) within +/- 0.03 mg/L. See Appendix C for experiment results and statistical analysis.

4.2.2 Tolyltriazole Influences

An experiment was conducted to ensure that tolyltriazole, at high concentrations, did not affect the probe membrane. Average DO uptake rates obtained from three microcosms containing no tolyltriazole and three microcosms containing 1000 ppm were compared to determine whether or not a statistical difference existed. The experiment covered the same time period as the toxicity experiments. The results show that the presence of tolyltriazole has no statistically significant influence at a 0.05 level of significance on the membrane. Reference Appendix D for experimental data and statistical procedures.

4.3 Microorganism Identification Lab Results

Lab results do not correspond with the results found during the initial classification efforts. According to the reports it is apparent that the microorganisms were not Gram-negative as originally thought. The lab results also indicate that the shape of the microorganisms is different than originally thought. According to the Microcheck, INC. results the Saturn and Mars microorganisms were both rod shaped. The final interesting finding is that the microbial colonies: Saturn, Mars and *Bacillus*, originally thought to be isolated were actually a consortia of microorganisms. Table 9 summarizes the results that can be found in Appendix A. Each lab uses a different identification procedure and for that reason may come up with different species and / or genus. The identification processes are highly variable. The labs report the most likely genus and

species, based on their identification procedure. Had two cultures been identified as the same, they would not necessarily behave in the same manner. The presence of other organisms may influence the behavior of the predominant species.

Table 9. Microbial Analysis - Summary

Culture	Laboratory / Method		
	Microbial Insights	**MIDI Labs / 16S rDNA**	**Microcheck / Fatty Acids**
Saturn	*-Streptococcus macrosporus* *-Microbacterium sp.*	*-Arthtrobacter crystallopoietes*	*-Bacillus disposauri* *-Arthrobacter protophormiae* *-Corynebacterium aquaticum*
Bacillus	*-Bacillus thuringiensis*	*-Bacillus cereus*	*-Bacillus cereus* *-Bacillus mycoides*
Mars	*-Staphlococcus warneri* *-Bacillus* – like *-Bacillus* – like *-Clostridium sp.*	*-Microbacterium chocolatum* *-Staphylococcus warneri*	*-Bacillus disposauri* *-Cellulomonas turbata*

4.4 Toxicity Analysis of Tolyltriazole on DO Consumption

The statistical tests were chosen in order to determine if a significant difference existed between the DO uptake rates at each concentration and that of the control (0 ppm). Visual analysis of the data indicated whether or not the data followed expected trends.

Graphs of DO uptake rates were created using each microbial population's data to visually inspect for trends or differences. Graphs of the DO rate at each tolyltriazole concentration were generated using the following method. The DO concentration at the beginning and end of a nine-minute period was recorded. The rate was calculated by

taking the difference of the two recorded values and dividing by the time period (9 minutes) as shown in the equation below:

$$DOuptakeRate = \frac{[DO_t] - [DO_{t+9}]}{9\,\text{min}}$$

The calculation was performed for each nine-minute interval for which data existed. Data was recorded to provide seven nine-minute time intervals. Initial evaluation of the graphs of each microbial population's DO uptake rate indicates that the microorganisms react differently when exposed to various concentrations of tolyltriazole.

An ANOVA was used to determine if significant statistical differences existed between the means indicating a need for further analysis. The Dunnett's comparison was used to determine if significant differences existed between the DO consumption rate of unexposed microorganisms (0 ppm) and the DO consumption rate of the microorganisms exposed to various tolyltriazole concentrations (50, 100, 500, and 1000 ppm).

4.4.1 ANOVA Test Results

The ANOVA analysis was performed using the statistical program JMP®. A P-value was found for each time interval and based on the P-value it was determined whether or not further statistical analysis was required. A P-value less than the α value results in rejection of the null hypothesis, stating that no statistical difference exists, and is denoted with a Y. A P-value grater than the α value prevents rejection of the null hypothesis, stating that a statistical difference does not exist, and is denoted with an N. Further statistical testing was only required for the intervals denoted with a Y, indicating

a significant difference existed, however Dunnett's comparison was performed on all points for a more detailed analysis. Reference Appendix E for actual P-values.

Table 10. Summary of ANOVA Results

Micro-organism	Time Interval						
	1	2	3	4	5	6	7
Saturn	Y	Y	Y	Y	Y	Y	Y
Bacillus	Y	Y	Y	Y	Y	Y	Y
Mars	N	N	N	Y	N	N	N/A

4.4.2 Saturn Evaluation

An experiment using the Saturn microorganism was conducted. A graph of the average DO uptake rates is shown in Figure 5. All DO uptake rate points are an average of three microcosms. See Appendix F for actual DO uptake rate values for each microcosm, the average DO uptake rate, and the sample standard deviation.

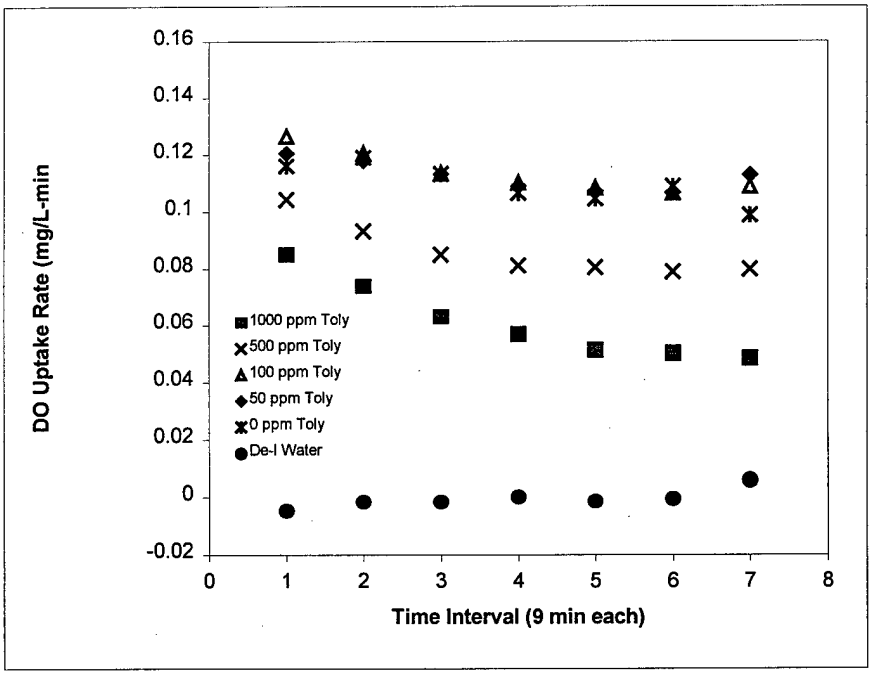


Figure 5. Saturn DO Consumption Rate

Visually it appears that there is no significant difference between the 0 ppm (control), 50 ppm, and 100 ppm microcosms. The 500 ppm and 1000 ppm average rates are lower than the others indicating that some inhibition has occurred. Whether the tolyltriazole killed microorganisms or merely prevented them from replicating cannot be determined using the current methodology. The de-ionized water line indicates that oxygen entering the experimental set-up was negligible.

In order to verify that the inoculum was well mixed, three 20 mL samples were taken, filtered and the dry weight recorded. A well-mixed sample would result in dry weight values having a small standard deviation. Three replicates were taken and the average and standard deviation were calculated. The results are shown in the table below. The small standard deviation indicates that the inoculum was well mixed.

Table 11. Saturn Dry Weights

Replicate #	Wt After Drying (g/L)	Wt After Burn (g/L)	Wt of Organics (g/L)	Wt of Organics (mg/L)
1	83.1500	83.2650	0.1150	115.0000
2	83.1550	83.2400	0.0850	85.0000
3	82.4000	82.4950	0.0950	95.0000
		Average	0.0983	98.3333
		Standard Dev	0.0153	15.2753

The ANOVA indicated that statistical differences existed among the means in all time intervals. Confirmation that the experimental means differ from the control mean is provided by the results of the Dunnett's analysis. A summary of the Dunnett's comparison findings is shown in the table below, where N indicates there is no significant difference from the control (0 ppm), and Y indicates a significant difference.

Table 12. Saturn Dunnett's Comparison Summary

Time Interval	Tolyltriazole Concentration			
	50 ppm	100 ppm	500 ppm	1000 ppm
1	N	Y	Y	Y
2	N	N	Y	Y
3	N	N	Y	Y
4	N	N	Y	Y
5	N	N	Y	Y
6	N	N	Y	Y
7	N	N	N	Y

Reference Appendix G for actual Saturn Dunnett's results including the comparison circles and the numerical difference between the difference of the two tested means and the critical difference.

4.4.3 *Bacillus* Evaluation

An experiment using the *Bacillus* microorganism was conducted. A graph of the average DO uptake rates is shown in Figure 6. All DO uptake rate points are an average of three microcosms. See Appendix H for actual DO uptake rate values for each microcosm, the average DO uptake rate, and the sample standard deviation.

The data for this microorganism does not follow any obvious trend. While the average DO uptake rate is lower for 1000 ppm, not all of the data points are consistent. One point of interest is the fact that the average of the DO consumption within the 500 ppm microcosms had the greatest average DO uptake rate. As with the Saturn microorganism the average DO uptake rates for the 0 ppm, 50 ppm, and the 100 ppm microcosms do not appear to be different. The inclusion of the de-ionized water DO

uptake rate shows that the oxygen entering the microcosms throughout the experiment was negligible.

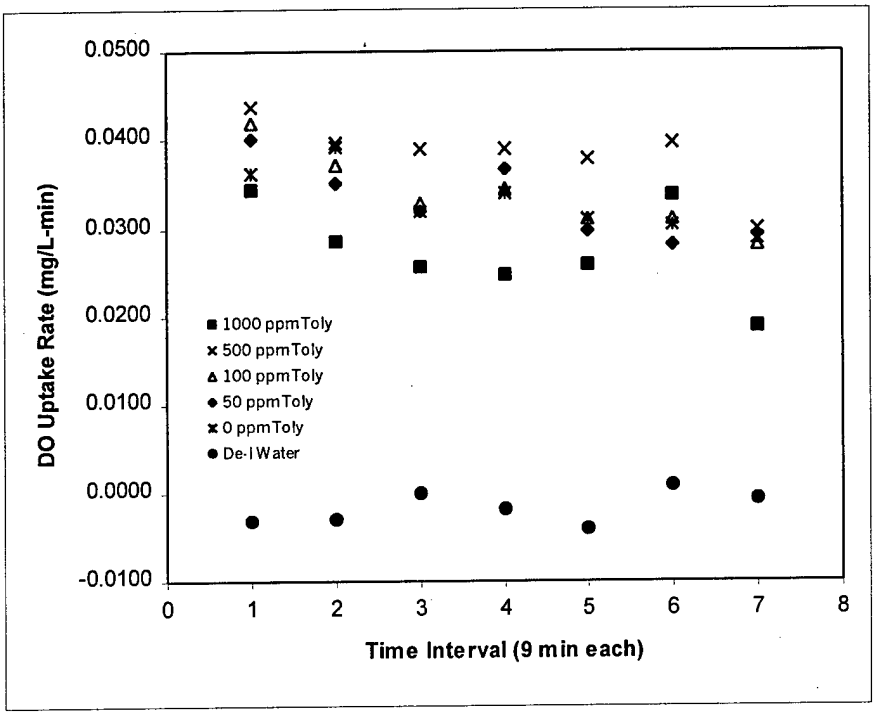


Figure 6. *Bacillus* DO Consumption Rate

In order to verify that the inoculum was well mixed three 20 mL samples were taken, filtered, dried and the dry weight recorded. A well-mixed sample would result in a small standard deviation. Three replicates were taken and the average and standard deviation were calculated. The results are shown in the table below. The small standard deviation indicates that the inoculum was well mixed.

Table 13. *Bacillus* Dry Weights

Relicate #	Wt After Drying (g/L)	Wt After Burn (g/L)	Wt of Organics (g/L)	Wt of Organics (mg/L)
1	82.2600	82.3850	0.1250	125.0000
2	83.1150	83.2750	0.1600	160.0000
3	82.4700	82.6400	0.1700	170.0000
		Average	0.1517	151.6667
		Standard Dev	0.0236	23.6291

The ANOVA indicated that statistical differences existed among the means within all the time intervals. Confirmation that the experimental means differ from the control mean is provided by the results of the Dunnett's comparison. The inconsistency of the data is further supported by the Dunnett's results. Differences from the control were not consistent for any set of data. Overarching conclusions on each concentration were difficult to make because of the inconsistency, however point-to-point comparisons are summarized below. A summary of the Dunnett's comparison findings is shown in the table below, where N indicates there is no significant difference from the control (0 ppm), and Y indicates a significant difference

Table 14. *Bacillus* Dunnett's Comparison Results

Time Interval	Tolyltriazole Concentration			
	50 ppm	100 ppm	500 ppm	1000 ppm
1	N	N	N	N
2	N	N	N	Y
3	N	N	Y	Y
4	N	N	N	Y
5	N	N	Y	Y
6	N	N	Y	N
7	N	N	N	Y

Reference Appendix I for actual *Bacillus* Dunnett's comparison data including the comparison circles and the numerical difference between the difference of the two tested means and the critical difference.

4.4.4 Mars Evaluation

An experiment using the Mars microorganism was conducted. A graph of the average DO uptake rates is shown in Figure 7. All DO uptake rate points are an average

of three microcosms. See Appendix J for actual DO uptake rate values for each microcosm, the average DO uptake rate, and the sample standard deviation.

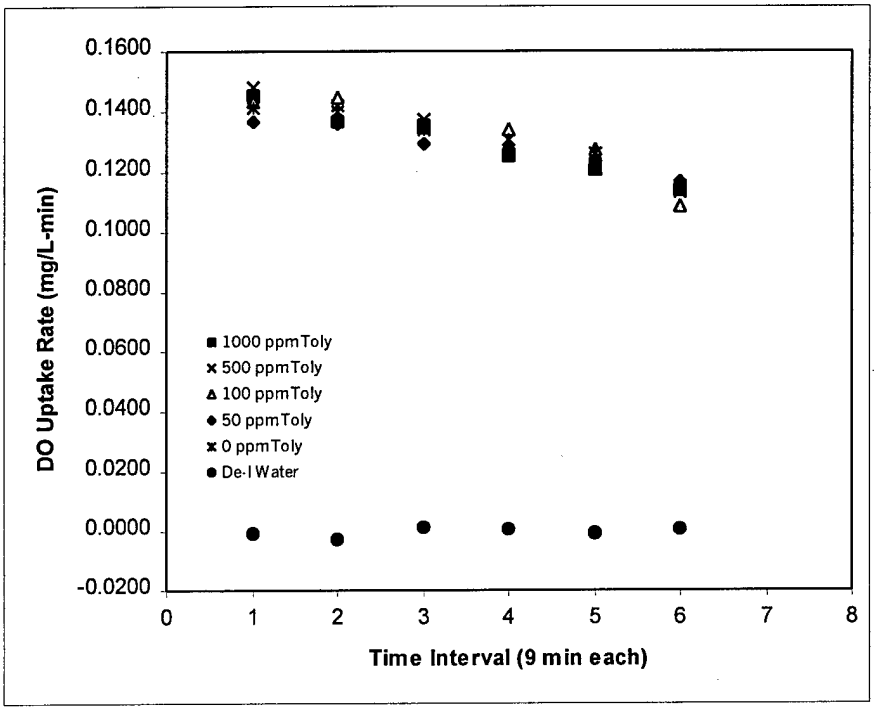


Figure 7. Mars DO Consumption Rate

The average DO consumption rates graphed in Figure 7 show no impact on the microorganisms as a result of the increasing tolyltriazole concentrations. Visually there appears to be no difference on the microorganism's capability to consume oxygen between the different concentrations. The inclusion of the de-ionized water DO uptake rate shows that the oxygen entering the microcosms throughout the experiment was negligible.

In order to verify that the inoculum was well mixed three 20 mL samples were taken, filtered and the dry weight recorded. A well-mixed sample would result in a small standard deviation. Three replicates were taken and the average and standard deviation

were calculated. The results are shown in the table below. The small standard deviation indicates that the inoculum was well mixed.

Table 15. Mars Dry Weights

Replicate #	Wt After Drying (g/L)	Wt After Burn (g/L)	Wt of Organics (g/L)	Wt of Organics (mg/L)
1	83.0600	83.2100	0.1500	150.0000
2	82.3250	82.4450	0.1200	120.0000
3	83.1600	83.3050	0.1450	145.0000
		Average	0.1383	138.3333
		Standard Dev	0.0161	16.0728

The ANOVA indicated that statistical differences existed only at time interval four. The Dunnett's comparison was performed on all time intervals, providing a more detailed analysis, to determine if any of the experimental means differed significantly from that of the control mean. The results of the Dunnett's comparison are presented in the table below and show that no statistical differences occur between any of the experimental means and that of the control mean. This indicates that the microorganism is not negatively impacted by the presence of tolyltriazole at the concentrations tested.

Table 16. Mars Dunnett's Comparison Results

Time Interval	Tolyltriazole Concentration			
	50 ppm	100 ppm	500 ppm	1000 ppm
1	N	N	N	N
2	N	N	N	N
3	N	N	N	N
4	N	N	N	N
5	N	N	N	N
6	N	N	N	N

Reference Appendix K for Mars Dunnett's comparison results including the comparison circles and the numerical difference between the difference of the two tested means and the critical difference.

4.5 Microorganism Rate Comparison

A visual comparison of the three microorganism's control rates indicates that there is a difference between each of the microorganisms. To ensure that the rates were standardized to some common factor, each was normalized to mass of microorganism (MO). The chart below graphically displays each microorganism's normalized control DO uptake rate. The coccus (Saturn and Mars) cultures had DO uptake rates that were greater than that of the rod-shaped (*Bacillus*, Jupiter, and Venus) cultures.

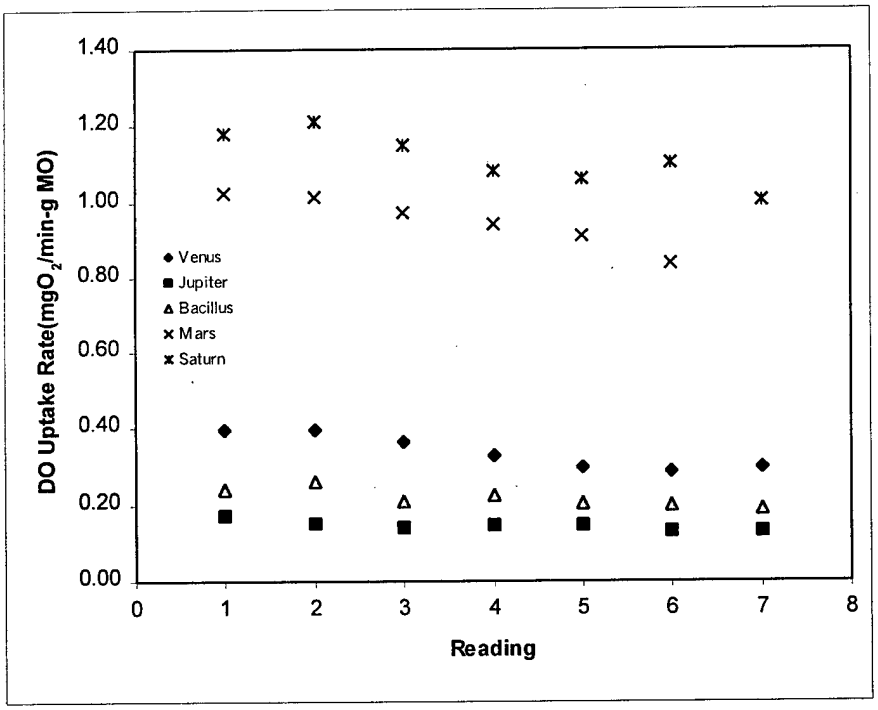


Figure 8. Normalized Microorganism Control DO Uptake Rate

The Jupiter and Venus cultures were evaluated by Leonard (2000). The same experimental methods and evaluation procedures were used in obtaining the DO consumption rates.

4.6 Microbial Plate Count Toxicity Test

The microbial colony count test used diluted samples from each microcosm collected at the end of each experimental run. The range of acceptable number of CFUs to constitute a valid test, as described in Standard Methods, is 30 to 300. Saturn and *Bacillus* resulted in a number of colonies that was too great to count. Mars, even though it fell outside of the acceptable range, was countable. The control (0 ppm) was used as the baseline. The data indicates that the increasing amounts of tolyltriazole affected the ability of the microorganism to replicate; an increasing concentration resulted in a decreasing number of colonies. Experimental counts and statistical test results can be seen in Appendix L.

A Dunnett's comparison of the plate count data indicates that only the 1000 ppm data is statistically different from that of the control. While 1000 ppm is the only comparison resulting in a statistical difference, the trend of the data indicates that as the concentration increases the ability of the microbial population to replicate decreases. Table 17 summarizes the colony counts as normalized to the control. A normalized value greater than one indicates that the microbial population replicated at a rate greater than the control. In this situation the normalized rate decreased as the concentration increased, indicating that the ability of the microorganisms to replicate decreased as they were exposed to higher concentrations of tolyltriazole.

Table 17. Mars Normalized Colony Counts

Concentration	Normalized Colony Count Values
0 ppm	1.00
50 ppm	0.83
100 ppm	0.88
500 ppm	0.85
1000 ppm	0.62

4.7 Potential Sources of Error

Several potential sources of error exist. Had there been no sources of error the standard deviation associated with averaging the three rates would be zero. For this thesis effort the standard deviation was assumed to be the total error of the experiment. Some of the error includes the sensitivity of the DO probes, the scales, and the glassware used to measure liquid volumes. Precautions were taken to ensure that deviations from the standard procedures were minimized to reduce sources of error.

V. Conclusions

5.1 Conclusions

The objective of this research was to study the toxicity of tolyltriazole on different microorganisms. Previous studies have shown tolyltriazole to be moderately toxic at high concentrations. The objective was to expand the knowledge base using isolated microorganisms and various concentrations of tolyltriazole.

A methodology was derived which produced reliable and consistent results. The methodology was easily implemented using the available resources. DO concentration values, the required data, were easily obtainable.

Three isolated microbial cultures were exposed to different concentrations of tolyltriazole and the DO concentration values recorded. Each of the microorganisms reacted differently to the various concentrations of tolyltriazole. At the two lowest concentrations, 50 and 100 ppm, none of the microorganisms demonstrated statistically significant inhibition. One notable exception was the first data point for the Saturn culture. A possible explanation is that the initial exposure affected the microorganism, but after a short time period, the microorganism recovered or became acclimated. Data collected by Leonard (2000) on rod shaped microorganisms (Jupiter and Venus) concurred with the results found in this thesis effort, no inhibition at 50 and 100 ppm.

The three cultures acted differently at 500 ppm. Saturn consumed DO at a lower rate, indicating inhibition. Mars showed no inhibition due to the tolyltriazole exposure. Saturn was significantly influenced at all data points except for the final data point. The

reason for the apparent tolerance at the final data point is not known, however one possible explanation would be that Saturn became acclimated to the tolyltriazole. *Bacillus*, on the other hand, had mixed results. Three of the seven data points were significantly different from the control, but the interesting point is that the three average DO consumption rates are greater than the control. Perhaps the tolyltriazole acted as a stimulant, increasing the rate of DO uptake. The other data points are not significantly greater. Microbial cultures studied by Leonard (2000) showed tolyltriazole acted as a stimulant at 500 ppm.

At 1000 ppm Saturn and *Bacillus* were inhibited, however, Mars showed no inhibition. Saturn showed significant inhibition at all data points. *Bacillus* DO consumption rates were significantly lower at all but two data points. Leonard's (2000) studies showed microbial inhibition at 1000 ppm on the Jupiter and Venus cultures.

It appears that the microorganisms (Saturn and *Bacillus*) are impacted at high concentrations ($\geq$ 500 ppm). Lower concentrations ($<$ 500 ppm) did not appear to inhibit microbial activity.

Useable plate count data was only obtained from the microorganism Mars. Based on the results of this test, the ability of the microorganism to replicate (form CFUs) was inhibited as the exposure to tolyltriazole increased. The data was normalized to the control 0 ppm and at 1000 ppm the normalized number of CFUs was 62% of the control.

The two microorganisms originally identified as Gram-negative and coccus (Mars and Saturn) had a greater DO consumption rate than the Gram-positive *Bacillus* (rod-shaped). Data collected by Leonard (2000) on Gram-negative rod shaped microorganisms resulted in lower DO consumption rates. Gram-negative

microorganisms tend to have an outer membrane that acts as a barrier to larger molecules. Perhaps the membrane was capable of slowing tolyltriazole's entrance into the cell.

5.2 Improvements

Several improvements could be made to ensure more consistent results. With the existing methodology, slight modifications and equipment upgrades would significantly improve the quality of the results.

5.2.1 Microorganism Cultivation

The use of a more consistent microorganism cultivation process would be the most beneficial improvement. Several of the inconsistencies are a result of the changing concentration of microorganisms placed into the growth solution. The use of a continuous bioreactor would have greatly improved the ability of to keep the number of microorganisms constant from experiment to experiment. The bioreator supplies a constant amount of food and removes a constant amount of microorganisms to maintain an environment of steady growth. The use of a bioreactor may also decrease the possibility of contaminating a pure culture.

5.2.2 Equipment Upgrades

The equipment used, especially the DO probes, was not best suited for the methodology decided upon. An automated recorder would enhance the process by allowing an increased number of experiments to be run for longer time periods. The number of errors in the recorded data would be reduced. A built in timer could be set to

ensure readings were consistently taken at the right interval, also preventing the possibility of missed readings.

5.3 *Follow-on Research*

Several potential research ideas came up throughout the duration of the experiment.

5.3.1 Increase the Range of Tolyltriazole Tested

Based on the data generated during this thesis effort, the 50 ppm and even the 100 ppm microcosms could be eliminated and the focus shifted to higher concentrations. Little impact was observed at the low concentrations. A more focused effort to look at the effect of concentrations between 100 and 500 ppm should be undertaken. Based on the generated data, the DO consumption decreased at the 500 ppm and not the 100 ppm indicating that the on set of toxicity occurs between the two concentrations.

5.3.2 Determine Residual Tolyltriazole Concentrations

A possible area of focus is on the tolyltriazole remaining in the microcosm after the experiment has been run. Perhaps the microorganisms are capable of degrading tolyltriazole to a certain degree even if the tolyltriazole initially inhibits microbial activity. In addition, one could determine if acclimation time has any influence on the microorganism's ability to degrade tolyltriazole or recover from initial inhibition.

5.3.3 Use of Luminescence

Luminescence studies are useful in determining the mechanism of toxicity, whether the chemical penetrates the microorganism or acts on the cell's surface. The

microorganisms will luminescence to a certain degree on their own. The degree of luminescence may or may not change upon exposure to tolyltriazole. Any noted difference, either an increase or decrease, could be used to focus on possible mechanisms of toxicity. The change in luminescence may prove to be a more accurate measure of toxicity.

Appendix A: Microorganism Identification Results

Microcheck, INC. Laboratory Analysis Results

LINE NO.	SAMPLE ID LABEL	✓	MICROORGANISM IDENTIFICATION	SI / SD	TYPE	MEDIA	CONFIRM TEST	LAB COMMENTS
1	Saturn		*Bacillus cereus* GC subgroup B MIXED CULTURE: see lines 1a, 1b, 1c below	0.463	B	TSBA		different colony types apparent after analysis
1a	Saturn.1 (large cream colony)	✓	*Bacillus disposauri*	0.747	B	TSBA		
1b	Saturn.2 (small yellow colony)	✓1	*Arthrobacter protophormiae/ramosus* *Paenibacillus polymyxa* *Bacillus megaterium* GC subgroup B	0.713 0.696 0.594	B	TSBA	GPR and cocco-bacilli	our spore stain result supports marked (1) FAME identification
1c	Saturn.3 (small gray colony)	✓	*Corynebacterium aquaticum*	0.682	B	CLIN		see Lab Notes at end of Results Table
2	Venus		*Xanthomonas axonopodis vitians* MIXED CULTURE: see lines 2a, 2b below	0.021	B	TSBA		different colony types apparent after analysis
2a	Venus.1 (opaque colony)	✓	*Bacillus coagulans*	0.350	B	TSBA		
2b	Venus.2 (translucent colony)	1	*Nesterenkonia halobia* *Kocuria varians* *Microbacterium lacticum*	0.633 0.497 0.382	B	TSBA	GPR	our stain result supports marked (1) FAME identification
3	Mars		*Bacillus cereus* GC subgroup B MIXED CULTURE: see lines 3a, 3b below	0.682	B	TSBA		different colony types apparent after analysis
3a	Mars.1 (large cream colony)	✓	*Bacillus disposauri*	0.719	B	TSBA		

LINE NO.	SAMPLE ID LABEL	✓	MICROORGANISM IDENTIFICATION	SI / SD	TYPE	MEDIA	CONFIRM TEST	LAB COMMENTS
3b	Mars.2 (small yellow colony)	1	*Nesterenkonia halobia* *Cellulomonas turbata* *Kocuria varians*	0.463 0.424 0.424	B	TSBA	GPR and cocco-bacilli	our stain result supports marked (1) FAME identification
4	Jupiter	✓	*Bacillus cereus* GC subgroup A	0.678	B	TSBA		
5	1		*Bacillus cereus* GC subgroup A MIXED CULTURE: see lines 5a, 5b below	0.691	B	TSBA		different colony types apparent after analysis
5a	1.1 (round colony)	✓	*Bacillus cereus* GC subgroup B	0.601	B	TSBA		
5b	1.2 (irregular colony)	✓	*Bacillus mycoides* GC subgroup B	0.406	B	TSBA		

Lab Notes:

Line No. 1c: The fatty acid profile of isolate Saturn.3 was compared to *Corynebacterium aquaticum*, a Gram positive rod which can be readily identified from growth on blood agar, and *Legionella brunensis*, a Gram negative rod which requires buffered charcoal'yeast extract (BCYE) agar for growth. Since these organisms were grown on blood agar at 35°C for one day *Corynebacterium aquaticum* represents a much better choice for the identity of this isolate.

For your benefit, please find a brief description of our METHODOLOGY, an interpretation of the SIMILARITY INDEX, an explanation of the COMPARISON CHART, the rationale for Gram staining (GRAM STAIN), spore staining (SPORE STAIN) and how fungi (FUNGI) are identified in our laboratory in the six paragraphs below.

METHODOLOGY - The microbial identification systems at MICROCHECK consist of gas chromatographs with flame ionization detectors, autosamplers, integrators, and computers. The identification systems use five percent methyl phenyl silicone capillary columns. Retention time (RT) stability to two thousandths of a minute permits automation of the fatty acid methyl ester (FAME) peak naming. Prior to commencing analyses, there are 2 microliter injections of commercial calibration standards into the gas chromatographs. Besides calibration prior to each new method the gas chromatographs are also recalibrated every 50 samples. Once the systems have been calibrated and are operational, the autosamplers inject the cellular fatty acid extracts. The integrators process the chromatographic data and send the data to the computers. The computers name the FAME peaks and compare the fatty acid profiles of the unknown organisms to the profiles of the

7000 strains in the databases. The comparison of the fatty acid profile of an unknown to those in the databases is accomplished through use of software which uses covariance matrix, principal component analysis, and pattern recognition. Due to the large number of fatty acids produced by aerobic and anaerobic bacteria, yeast, and actinomycetes and the uniqueness of one pattern for a given taxon, the identification is very accurate. In addition to the internal standardization with the calibration fluid, an external control, *Stenotrophomonas maltophilia*, is also used. This bacterium, which has 23 fatty acid peaks, is processed daily from the streak plates with the client unknowns.

SIMILARITY INDEX - On the computer printout the similarity indices (SI) appear below the printout of the fatty acid composition and above the comparison chart in which the fatty acids of an unknown microorganism are compared to those of the reference strains. The SI value is the number following the species name (and often subspecies or pathovar name) of the library entry to which the unknown is compared. The SI value is unrelated to probability ratios and although the values range from 0.001 to 0.999 it is not a percentage. The SI is a numerical value which expresses how closely the fatty acid composition of an unknown corresponds to the fatty acids of the strains used to generate the library entry. Each library entry represents the profiles of at least 15 different microbial strains from both clinical and environmental sources. If a particular fatty acid departs slightly from the mean of the library entry either in RT or percentage the SI drops from an initial value of 1.00. A SI of 0.500 to 0.999 is an excellent match, as well as a single match comparison (no other matches listed) of 0.300 and higher. For SI between 0.100 and 0.300 the species of the unknown may not be in the current version of the database but the genus is probably correct and for SI below 0.100 the genus of the unknown is also questionable. A NO MATCH indicates there are no close comparisons to the 7000 strains in the databases. The value of the comparison is expressed in standard deviations (SD) from the mean of the strains used to generate this distantly related database entry. Infrequently an organism cannot be compared to any entry in the databases. This also results in a NO MATCH but there is not a SD measurement.

COMPARISON CHART - The comparison chart is below the results of the library search on the computer printout. The name of the library entry to which the unknown is compared is in the upper left hand corner of the chart. In the upper right hand corner of the chart is the SD that the unknown is from the mean of the strains used to generate the library entry. In the chart the x's represent the percentage of each fatty acid from the unknown, the -'s represent the range of percentages for the library entry strains, the +'s represent the mean percentage for the library entry strains, and the *'s represent peak matches of the fatty acids of the unknown with the mean of the library entry.

GRAM STAIN - A Gram stain is done on an isolate that is compared to database entries with similarity indices that are within 0.300 of each other if the cellular morphologies of the database entries are different, that has a similarity index less than 0.100, or that is a NO MATCH to any of the database entries to determine if the morphology of the isolate is consistent with that of the comparison.

SPORE STAIN - A spore stain is done, rather than a Gram stain, if the fatty acid profile of an isolate is compared to database entries with similarity indices that are within 0.300 of each other and at least two of these comparisons are G^+ rods, one of which forms endospores.

FUNGI - Fungi are subcultured on plugs of potato flake agar (PFA) under coverslips on separate plates of potato dextrose agar (PDA) and on separate plates of phosphate glucose agar (PGA). Following incubation at 28°C the organisms are examined microscopically and identified using several keys to the fungi. Fungi do not have a SI / SD because they are not identified by FAME analysis.

FAME ANALYSIS Automated fatty acid methyl ester (FAME) analysis by gas liquid chromatography for identification of aerobic and anaerobic bacteria, yeast, and actinomycetes.

✓ A check mark next to a microorganism name indicates an excellent FAME match (See SI and SD)

SI The Similarity Index (SI) is a value between 0.001 and 0.999 which expresses the FAME similarity between the unknown isolate and the database match.

0.500 to 0.999	excellent match for genus and species
0.300 to 0.999	excellent for a single match to genus and species
0.100 to 0.300	good match for genus
0.001 to 0.099	weak match for genus

NO MATCH A NO MATCH analysis occurs when the unknown isolate has no close comparisons in the database.

SD The Standard Deviation (SD) value is listed for a NO MATCH analysis. The SD is a mathematical expression of the distance between the fatty acid profile of the unknown and the mean profile of the closest database entry. A NO MATCH with no SD indicates that the microorganism was not even distantly related to any of the 2,000 entries in the databases.

TYPE Microorganism TYPE

AC	actinomycete	FAN	facultative anaerobe
AN	anaerobic bacterium	M	mycobacterium
B	aerobic bacterium	TH	thermophilic bacterium
F	fungus	Y	yeast

( ) Parentheses ( ) around an entry in the TYPE column indicates that the microorganism was a different type than listed on the Test Request Form.

MEDIA The subculture MEDIA used by our laboratory to grow microorganisms.

BHIA	brain heart infusion blood agar	PYGT	peptone-yeast-glucose broth w/ Tween 80
CLIN	blood agar	R2A	defined minimal nutrient agar
MB7	Middlebrook 7H10 agar	SDA	Sabouraud dextrose agar
MRSA	MRS *Lactobacillus* agar	TSB	trypticase soy broth
PPP	potato dextrose agar, and phosphate glucose agar	TSBA	trypticase soy broth agar
PYG	peptone-yeast-glucose broth		

( ) Parentheses ( ) around an entry in the MEDIA column indicates that growth of the microorganism on this medium was insufficient for analysis.

CONFIRM TEST CONFIRMATION TESTING is done on an isolate whose FAME analysis result is inconclusive.

1 = stain 2 = coagulase test 3 = oxidase test 4 = API 20E test

CONFIRM TEST RESULTS

GPR	Gram positive rods	GPC	Gram positive cocci
GNR	Gram negative rods	GNC	Gram negative cocci
GVR	Gram variable rods		
coag$^+$	coagulase positive	coag$^-$	coagulase negative
ox$^+$	oxidase positive	ox$^-$	oxidase negative

API 20E Metabolic characterization which is done to confirm FAME results for members of the family Enterobacteriaceae. API 20E results often provides a more reliable identification than FAME analysis because this group of microorganisms is very similar in their FAME.

We encourage you to call our Technical Manager with any questions you may have about the analyses or the results:
Dr. Mike Sinclair - 802/485-6600 @ ext.22

AFIT/ENV

MOLECULAR ANALYSIS VIA 16S rDNA-DGGE

JANUARY 11, 2000

SUMMARY

The bacterial communities from 5 cultures from soils were characterized by denaturing gradient gel electrophoresis (DGGE). Results from the DNA profiles revealed both similarities and differences with their composition. All of the dominant bands excised from these samples fell within the Gram positive phylum. Several bands (B, C, D, and L) appeared to be present within more than one sample. Identification of individual bands are found in Table 1.

OVERVIEW:

The denaturing gradient gel electrophoresis (DGGE) approach directly determines the species composition of complex microbial populations based on the amplification of 16S rDNA fragments in polyacrylamide gels containing a linearity-increasing gradient of denaturants. DNA fragments of the same length but with different base-pair sequences are separated based on their melting behavior in an polyacrylamide gel. The banding patterns and relative intensities of the recovered bands provide a measure of changes in the community. Dominant species, which compose at least 1% of the total community in order to remain above the background level of minor bacterial amplification products, can be excised and sequenced. Fine scale sequence analysis of individual bands are used to infer the identity of the source organisms based upon database searches and phylogenetic methods (1-4).

METHOD:

Nucleic acid extraction was performed using a bead-beating system adapted from Borneman *et al.*, with modifications (4). Sodium phosphate buffer, chaotropic reagent, glass beads, and sample were agitated in a microcentrifuge tube using a high speed bead beater. The sample mixture was centrifuged and the supernatant was collected. Chloroform was added, mixed thoroughly, and centrifuged. The aqueous supernatant was collected and combined with the first supernatant fraction. DNA was precipitated from the aqueous phase with an equal volume of isopropanol in an ice bath. DNA was pelleted by centrifugation, washed with 80% ethanol, air-dried, and re-dissolved in TE buffer. The DNA extract was purified by extraction twice with an equal volume of phenol/chloroform/isoamyl alcohol, followed by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer.PCR amplification of 16S rDNA gene fragments was performed as described in (5) with modifications. Thermocycling consisted of 35 cycles performed on a "Robocycler™" PCR block. DGGE employed a D-Code 16/16 cm gel system maintained at a constant temperature in TAE buffer. Gradients were formed between 20-55 denaturants with the gels stained in ethidium bromide. Gel images were captured by use of an Alpha Imager™ system. The central portion of bands of were excised and soaked in purified water. The re-amplified products were purified by gel electrophoresis and isolated by use of a Gene-Clean™ kit. Purified DNA was sequenced with an ABI-Prism automatic sequencer. Sequence identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

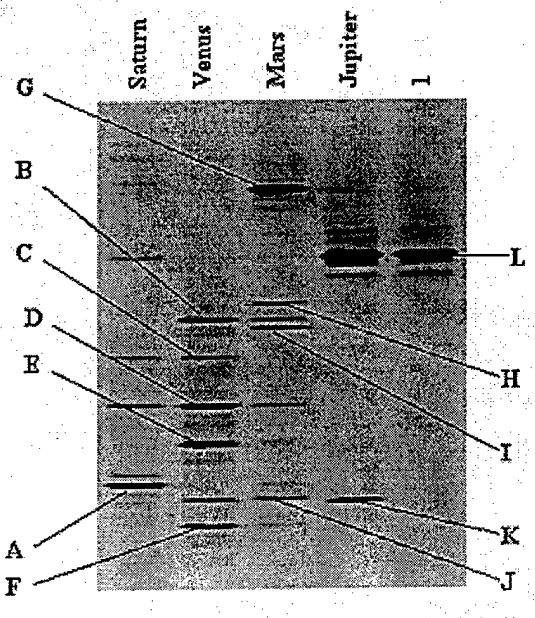


Figure 1. DGGE Gel Image.

Table 1. Sequence Results.

Band	Best Match	% Match	Phylogenetic affiliation	Ref. #
A	*Streptococcus macrosporus*	97%	Gram Positive Phylum	6
B	*Clostridium sp.*	100%	Gram Positive Phylum	-
C	Failed	-	-	-
D	*Microbacterium sp.*	95%	Gram Positive Phylum	-
E	*Uncultured actinomycete*	98%	Gram Positive Phylum	-
F	Failed	-	-	-
G	*Staphlococcus warneri*	100%	Gram Positive Phylum	-
H	*Unidentified eubacterium clone BSV45*	100%	Bacillus Like	7
I	*Unidentified eubacterium anoxic soil clone BSV77*	98%	Bacillus Like	7
J	Failed	-	-	-
K	Failed	-	-	-
L	*Bacillus thuringiensis*	100%	Gram Positive Phylum	-

Midi Labs Laboratory Analysis Results

Enclosed are your sample results, including 16S rRNA gene alignment profiles and phylogenetic tree displays.

The bacterial identifications assigned in this report are based on 16S rRNA gene sequence similarity. Sequences analysis was preformed using PE Applied Biosystem's MicroSeq™ microbial analysis software and database. The top ten alignment matches are presented in a percent genetic distance format. In this format a low percent indicates a close match.

Also provided with the report are neighbor joining (Saitou and Nei, **Mol. Biol. Evol.** 4(4):406-425, 1987) phylogenetic trees. The trees are generated using the top ten alignment matches.

Concise alignments are also included. These illustrate positions that differ between your sample and the first match in the database. The position of the mismatch is read vertically from top to bottom and the sequences are read horizontally from left to right.

The results provided in this report are intended for research use only and will be kept confidential.

The protocol used to generate the 16S rRNA gene sequence data is as follows:

The 16S rRNA gene was PCR amplified from genomic DNA isolated from bacterial colonies. Primers used for the amplification correspond to E. coli positions 005 and 1540 (full length packages) and 005 and 531 (500 bp packages). Amplification products were purified from excess primers and dNTPs using Microcon 100 (Amicon) molecular weight cut-off membranes and checked for quality and quantity by running a portion of the products on an agarose gel.

Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum and frozen at -20 °C until ready to load. Samples were resuspended in a solution of formamide/ blue dextran/ EDTA and denatured prior to loading. The samples were electrophoresed on a ABI Prism 377 DNA Sequencer. Data was analyzed using PE/Applied Biosystems DNA editing and assembly software.

Thank you very much for choosing MIDI Labs for your bacterial identification needs. Do not hesitate to contact MIDI Labs should you have any questions or comments concerning the data reports.

Please keep us in mind for your future identification or sequencing needs.

Identification Report Summary

Bacterial identifications assigned by MIDI Labs are based on 16S rRNA gene sequence similarity. Sample sequences are compared against PE Applied Biosystem's MicroSeq™ database using MicroSeq sequence analysis software. The top ten alignment matches are presented in a percent genetic distance format, which is basically the percent difference between two aligned sequences. This percentage takes into account any mismatched basepairs, gaps and IUB ambiguity codes. In this format a low percent indicates a close match.

Species Level - This indicates a species level match. A 16S rRNA sequence homology of greater than 99% is indicative of a species level match (Stackebrandt and Goebel). Our experience in developing the MicroSeq database leads us to agree with this conclusion, though we feel that there is no exact cut off point that can be applied to all groups. It is our opinion that each alignment needs to be analyzed individually, taking into account the percent genetic distance between known species within that group.

Note that our results are presented in a genetic distance format, which is essentially the opposite of percent homology.

Genus Level - This indicates that the sample appears to group within a particular genus but the alignment did not produce a species level match. A genus level match indicates that the sample species is not included in the MicroSeq database

No Match - This indicates that sample did not group well within any particular genus found in the MicroSeq database. In cases such as this, we search the GenBank and Ribosomal Database Project (RDP) databases with the sample sequence to try to provide a closer match. If the sample sequence does not match well with either of these databases, it may be a new species or a species whose 16S rRNA gene sequence is not present in any of the databases.

Reference:

Stackebrandt, E. and Goebel, B. M. 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. Int. J. Syst. Bacteriol. 44:846-849

Customer: Bleckmann

1/3/2000

MIDI LABS

500 bp Identification Summary

MicroSeq Database

C code	sample	closest match	% difference	confidence level
N267BLE	C2876 Saturn con	Arthrobacter crystallopoietes	1.98 %	Genus
N267BLE	C2878 Mars con	Microbacterium chocolatum	2.98 %	Genus
N267BLE	C2879 Jupiter con	Bacillus thuringiensis	0.00 %	Species
N267BLE	C2880 1 con	Bacillus cereus	0.65 %	Species
N267BLE	C2898 Mars white con	Staphylococcus warneri	0.19 %	Species

Key:

* - See report for additional comments concerning this field.

C code - Customer number assigned by MIDI Labs.

sample - Sample number assigned by MIDI Labs, followed by name assigned by customer.

closest match - Closest match to sample when aligned in a pairwise manner against the MicroSeq Database.

% difference - Percent difference between the sample and the closest match. Mismatched basepairs, gaps, and ambiguity codes are all accounted for in this percentage.

confidence level - This indicates the level of identification; see Identification Report Summary for additional information.

For research use only

Customer: Bleckmann

MIDI LABS

/3/2000

Partial 16S rRNA Gene Alignment with GenBank & RDP Databases

ample #	closest GenBank match	% ID	closest RDP match	SR
2876 Saturn con	Arthrobacter globiformis	97%	Arthrobacter crystallopoietes	.88
2878 Mars con	Aureobacterium kitamiense	97%	Aureobacterium testaceum	.83

- Customer number assigned by MIDI Labs.

ample # - Sample number assigned by MIDI Labs, followed by name assigned by customer.

losest GenBank match - Results from blast search of GenBank database.
ID - percent identity; this is essentially the percent similarity.

ee GenBank web page (www.ncbi.nlm.nih.gov/BLAST/blast_help.html)
r more detailed information.

losest RDP (Ribosomal Database Project) match - Results from search of RDP database.
R - similarity rank is given, we have observed that a ranking > .95 indicates species match.

ee RDP web page (rdpwww.life.uiuc.edu/rdphome.html) for more detailed information.

For research use only

Appendix B: Statistical Procedure for Performing Dunnett's Comparison

The information that is provided in the pages of this appendix explains the procedures and theory behind the Dunnett's comparison. The analysis determines if a statistical difference exists between a specified control mean and the selected data set mean. A two-tailed Dunnett's comparison was performed using a significance level of α=0.05.

Ho: No effect on the rate when exposed to some concentration of tolyltriazole
Ha: The rate was influenced (inhibited/enhanced) when exposed to tolyltriazole

$$H_o: \mu_{control} = \mu_{experiment}$$
$$H_a: \mu_{control} \neq \mu_{experiment}$$

A t-test statistic (t_d) is computed between each experimental group and the control using the following formula (Sheskin 362):

$$t_d = \frac{M_i - M_c}{\sqrt{\frac{2 * MSE}{n_h}}}$$

Where:
M_I = Mean of the experimental group
M_c = Mean of the control group
MSE = Mean Square Error
n_h = Harmonic mean of the sample sizes

$$n_h = \frac{k}{\frac{1}{n_1} + \frac{1}{n_2} + \cdots + \frac{1}{n_k}}$$

Where: k = Total treatment means, including control (Sheskin 363)

Three methods of comparison exist to determine if there is a statistical difference. The first is to compare the calculated t_d to the t_{crit}. The t_d was compared to the $t_{d(k,dfwG)}$ to determine whether or not the experiment mean was different than the control mean. The t-test (t_d) was calculated using each experimental point compared to the same experimental point in the control data. The t_{crit} was found in a table of Dunnett's critical t values in Sheskin page 699, based on the parameters of the experiment. The criterion for significant effect is shown below:

$td \geq t_{d(k,dfwG)}$ Significant Effect

The second comparison option is to compare the calculated critical difference to the difference between the experimental and control means (referred to as Abs(Dif) in the JMP® statistical software package). CD_d is the minimum required difference needed for the two means to differ significantly (referred to as Least Significant Difference (LSD) in JMP® statistical software package). The equation used to calculate the critical difference is shown below (Sheskin 363). This value is different for each comparison.

$$CD_d = t_{d(k,df_{WG})} * \sqrt{\frac{2 * MSE}{n_h}}$$

Where: $t_{d(k,dfwG)}$ = The tabled critical Dunnett value (2.89)
df_{WG} = Degrees of Freedom within the group

The criterion for significant effect is shown below:

$M_I - M_c \geq CD_d$ Significant Effect

The third method is to look at the comparison circles. The statistical program, JMP®, generated comparison circles for the generated data. An intercept of two specified comparison circles that results in an angle greater than 90° indicates that there is no statistical difference. An angle equal to 90° indicates a slight statistical difference, whereas an angle less than 90°indicates the means are statistically different.

Appendix C: Consistency Test Results

The table shown below reports the DO concentration recorded by each probe at the different levels of Na_2SO_3. The difference between the recorded values was calculated. The average of the differences and the standard deviation are the degree to which the two probes differ.

Table 18. Consistency Results

Na_2SO_3 Conc (gram)	DO Value (YSI DO Meter 58) (mg/L)	DO Value (YSI DO Meter 5100) (mg/L)	Difference (mg/L)
0.000	7.190	7.140	0.050
0.005	5.110	5.030	0.080
0.010	4.070	4.000	0.070
0.015	1.230	1.180	0.050
0.020	3.370	3.290	0.080
0.025	0.010	0.010	0.000
		Average	0.055
		Std Dev	0.030

The YSI DO Meter 58 consistently recorded higher DO values throughout the experiment.

Appendix D: Tolyltriazole Influence on DO Membranes

A two-sample t-test was performed using a significance level of α=0.05. Both populations were assumed normal and the two population variances were assumed equal.

Ho: No effect on the DO probe membrane when exposed to high concentrations of tolyltriazole

Ha: There was an effect on the DO probe membrane as a result of the tolyltriazole

The pooled estimator, an estimator of the common population variance, was determined using the following equation (Devore 358):

$$S_p^2 = \frac{(n_1 - 1) * S_1^2 + (n_2 - 1) * S_2^2}{(n_1 + n_2) - 2}$$

Where n_1 and n_2 represent the sample size of the respective data sets, and S_1 and S_2 are the standard deviation of the respective data sets.

The Standard error was determined using the following equations (Devore 358):

$$Std - Error = S_p \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

The calculated t-statistic (t) was calculated by dividing the difference of the means by the standard error:

$$t = \frac{\left(\overline{X}_{tolyltriazole} - \overline{X}_{control}\right)}{(Std - Error)}$$

The t-critical (t_{crit}) was determined for a two-tailed t-test because the effects of the tolyltriazole could have either enhanced or inhibited the performance of the membrane, as stated in the alternate hypothesis, therefore $\alpha/2$ was used.

Ho: $\mu_{control} = \mu_{tolyltriazole}$

Ha: $\mu_{control} \neq \mu_{tolyltriazole}$

$t_{crit} = t_{\alpha/2,n1+n2-2} = 2.776$ (Devore 707)

Given: $\alpha = 0.05$ (Level of Significance)

$n_1 = 3$ (number of control microcosms)

$n_2 = 3$ (number of tolyltriazole microcosms)

The t_{crit} was compared to the t statistic to determine whether or not the tolyltriazole had an impact on the membrane. An example of the criteria used to determine that there was no effect is shown below.

$T \leq -t_{crit}$	Inhibition
$t \geq t_{crit}$	Enhancement

The data listed below shows the values recorded during the experiment within each microcosm. The data was analyzed to determine if the presence of tolyltriazole had an impact on the membranes of the DO probe. The test showed that the presence of tolyltriazole had no effect on the membrane.

Table 19. Experimental Results

Time (Minutes)	Bottle 1 0 ppm	Bottle 2 1000 ppm	Bottle 3 0 ppm	Bottle 4 1000 ppm	Bottle 5 0 ppm	Bottle 6 1000 ppm
0	7.19	7.27	7.16	7.28	7.17	7.31
7	7.27	7.35	7.24	7.33	7.24	7.37
6	7.30	7.36	7.25	7.36	7.26	7.39
8	7.28	7.37	7.25	7.36	7.26	7.39
6	7.28	7.35	7.27	7.36	7.26	7.40
6	7.31	7.39	7.26	7.37	7.27	7.43
6	7.33	7.36	7.27	7.38	7.29	7.43
6	7.30	7.36	7.28	7.38	7.26	7.38
6	7.27	7.35	7.26	7.39	7.27	7.39
6	7.28	7.38	7.29	7.39	7.29	7.41
57	0.09	0.11	0.13	0.11	0.12	0.10

The final row is the difference between the initial and final reading. This value was divided by the total time (57 minutes) to get an overall rate of the total DO concentration change. The DO uptake rate is shown below for each microcosm.

Table 20. Average DO Drift

	Bottle 1 0 ppm	Bottle 3 0 ppm	Bottle 5 0 ppm	Bottle 2 1000 ppm	Bottle 4 1000 ppm	Bottle 6 1000 ppm
Average	0.0016	0.0023	0.0021	0.0019	0.0019	0.0018

Average 0 ppm	1.99E-03
Average 1000 ppm	1.87E-03

The results of the two-sample t-test are summarized in the table below.

Table 21. Results of Two-Sample T-Test

Mean 0 ppm	Std Dev 0 ppm	Mean 1000 ppm	Std Dev 1000 ppm	Pooled Estimator S_p^2	Standard Error	X_{Ctrl}-$X100_{0ppm}$	Calc T Value t_{Crit}=2.447	Upper Tail Test	Lower Tail Test
1.99E-03	3.65E-04	1.87E-03	1.01E-04	7.18E-08	2.19E-04	1.17E-04	5.35E-01	No Effect	No Effect

Appendix E: Summary of P-Values Found During ANOVA

A summary of the P-values found during the ANOVA analysis is presented below. A P-value less than the specified α value (0.05) indicates that there is a significant difference at a 0.05 level of significance. A P-value greater than the specified alpha indicates that there is not a significant difference between the means.

Table 22. Summary of ANOVA P-Values

Micro-organism	Time Interval						
	1	2	3	4	5	6	7
Saturn	.0001	.0001	.0001	.0001	.0001	.0001	.0001
Bacillus	.0288	.0013	.0011	.0037	.0005	.0076	.0213
Mars	.1927	.2431	.1042	.0151	.1108	.0802	N/A

ANOVA may indicate that there is a significant difference, however, further statistical analysis may indicate the a significant difference does not exist under the conditions specified by the additional analysis.

Appendix F: Saturn DO Uptake Rate Data

Time Interval (9 min)	Control Microcosm 1 (mg/L-min)	Control Microcosm 2 (mg/L-min)	Control Microcosm 3 (mg/L-min)	Control Mean (mg/L-min)	Control Std Dev	Control Std Error
1	0.1178	0.1133	0.1167	0.1159	0.0023	0.0013
2	0.1267	0.1156	0.1144	0.1189	0.0068	0.0039
3	0.1200	0.1089	0.1100	0.1130	0.0061	0.0035
4	0.1133	0.1033	0.1022	0.1063	0.0061	0.0035
5	0.1100	0.1022	0.1011	0.1044	0.0048	0.0028
6	0.1111	0.0967	0.1178	0.1085	0.0108	0.0062
7	0.1111	0.1011	0.0833	0.0985	0.0141	0.0081

Time Interval (9 min)	50 ppm Microcosm 1 (mg/L-min)	50 ppm Microcosm 2 (mg/L-min)	50 ppm Microcosm 3 (mg/L-min)	50 ppm Mean (mg/L-min)	50 ppm Std Dev	50 ppm Std Error
1	0.1233	0.1222	0.1156	0.1204	0.0042	0.0024
2	0.1167	0.1200	0.1167	0.1178	0.0019	0.0011
3	0.1067	0.1189	0.1133	0.1130	0.0061	0.0035
4	0.1056	0.1133	0.1078	0.1089	0.0040	0.0023
5	0.1000	0.1111	0.1100	0.1070	0.0061	0.0035
6	0.0989	0.1111	0.1100	0.1067	0.0068	0.0039
7	0.1011	0.1111	0.1256	0.1126	0.0123	0.0071

Time Interval (9 min)	100 ppm Microcosm 1 (mg/L-min)	100 ppm Microcosm 2 (mg/L-min)	100 ppm Microcosm 3 (mg/L-min)	100 ppm Mean (mg/L-min)	100 ppm Std Dev	100 ppm Std Error
1	0.1311	0.1222	0.1256	0.1263	0.0045	0.0026
2	0.1156	0.1233	0.1222	0.1204	0.0042	0.0024
3	0.1156	0.1078	0.1167	0.1133	0.0048	0.0028
4	0.1100	0.1078	0.1122	0.1100	0.0022	0.0013
5	0.1056	0.1067	0.1122	0.1081	0.0036	0.0021
6	0.1044	0.1033	0.1111	0.1063	0.0042	0.0024
7	0.1044	0.1044	0.1167	0.1085	0.0071	0.0041

Time Interval (9 min)	500 ppm Microcosm 1 (mg/L-min)	500 ppm Microcosm 2 (mg/L-min)	500 ppm Microcosm 3 (mg/L-min)	500 ppm Mean (mg/L-min)	500 ppm Std Dev	500 ppm Std Error
1	0.1056	0.1022	0.1044	0.1041	0.0017	0.0010
2	0.0922	0.0944	0.0922	0.0930	0.0013	0.0007
3	0.0867	0.0844	0.0833	0.0848	0.0017	0.0010
4	0.0811	0.0822	0.0800	0.0811	0.0011	0.0006
5	0.0811	0.0789	0.0811	0.0804	0.0013	0.0007
6	0.0800	0.0789	0.0778	0.0789	0.0011	0.0006
7	0.0800	0.0789	0.0800	0.0796	0.0006	0.0004

Time Interval (9 min)	1000 ppm Microcosm 1 (mg/L-min)	1000 ppm Microcosm 2 (mg/L-min)	1000 ppm Microcosm 3 (mg/L-min)	1000 ppm Mean (mg/L-min)	1000 ppm Std Dev	1000 ppm Std Error
1	0.0844	0.0844	0.0856	0.0848	0.0006	0.0004
2	0.0744	0.0733	0.0733	0.0737	0.0006	0.0004
3	0.0644	0.0611	0.0633	0.0630	0.0017	0.0010
4	0.0556	0.0578	0.0578	0.0570	0.0013	0.0007
5	0.0533	0.0489	0.0522	0.0515	0.0023	0.0013
6	0.0500	0.0500	0.0511	0.0504	0.0006	0.0004
7	0.0478	0.0478	0.0500	0.0485	0.0013	0.0007

Appendix G: Saturn Dunnett's Comparison Results

The graphs below have been generated by JMP®, the statistical program used to perform the Dunnett's comparison. The left side of the figure shows the three data points as represented with the three smaller dots. The larger darker dot represents the mean and the bar represents one standard error above and below the mean.

The circles to the right are the comparison circles. By looking at the circles one can determine if there is a significant difference or not between the each sample and the control. The circles' centers line up vertically and are also aligned with its group mean. The radius of the circle represents the 95% confidence interval of the group mean. The different angles of intersection (described in Appendix B) dictate whether there is a significant difference.

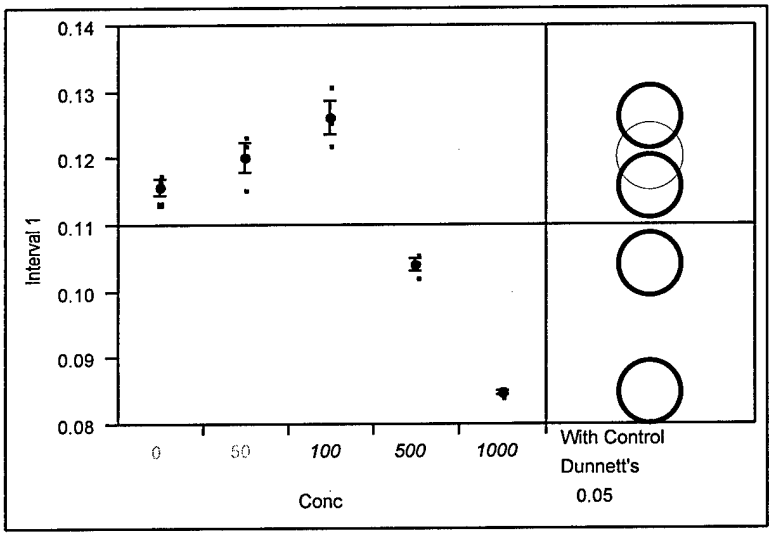


Figure 9. Saturn Interval 1

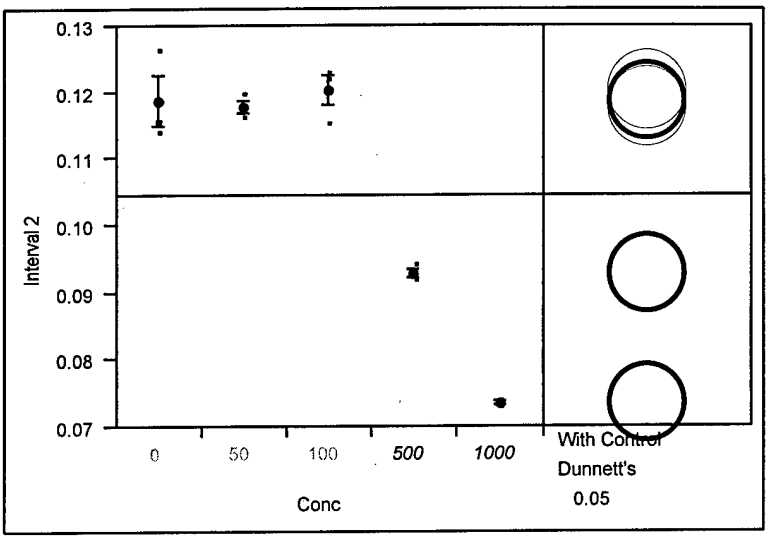


Figure 10. Saturn Interval 2

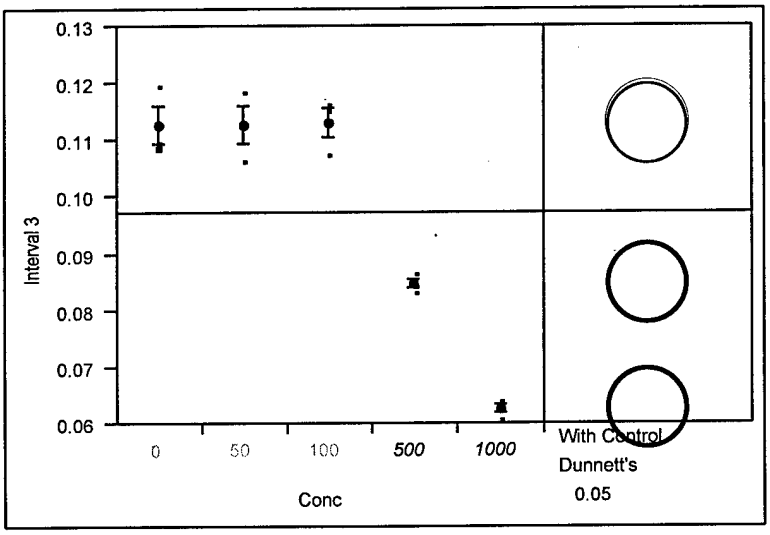


Figure 11. Saturn Interval 3

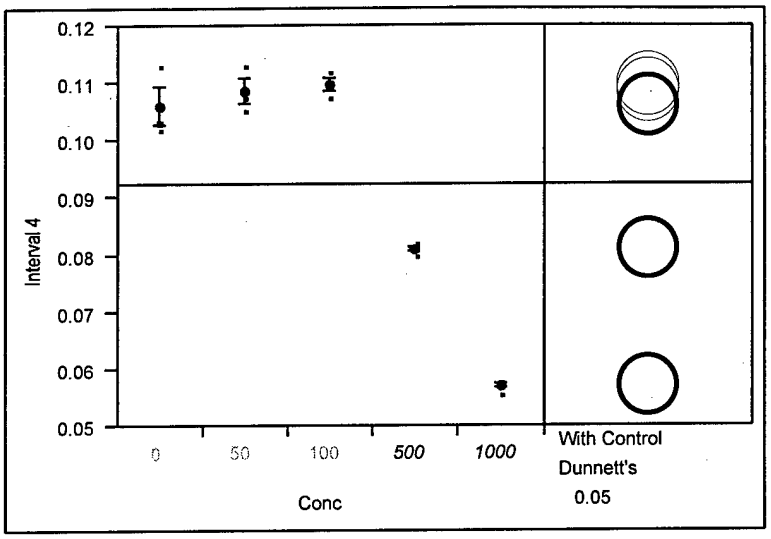


Figure 12. Saturn Interval 4

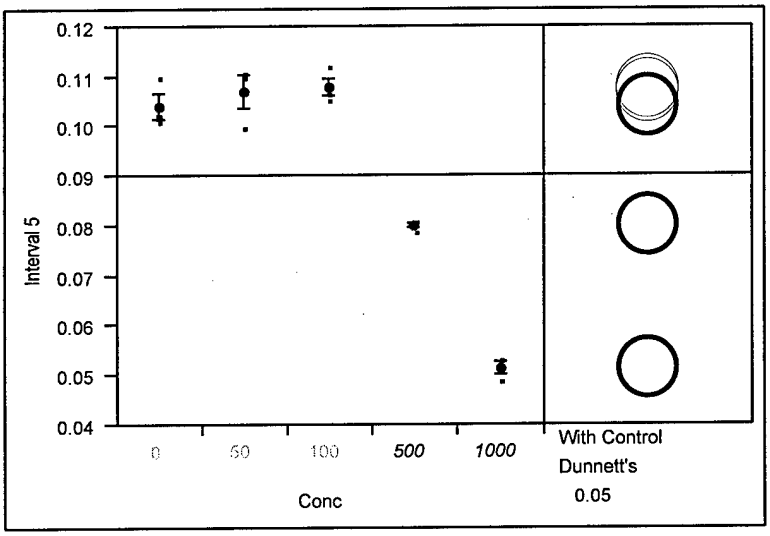


Figure 13. Saturn Interval 5

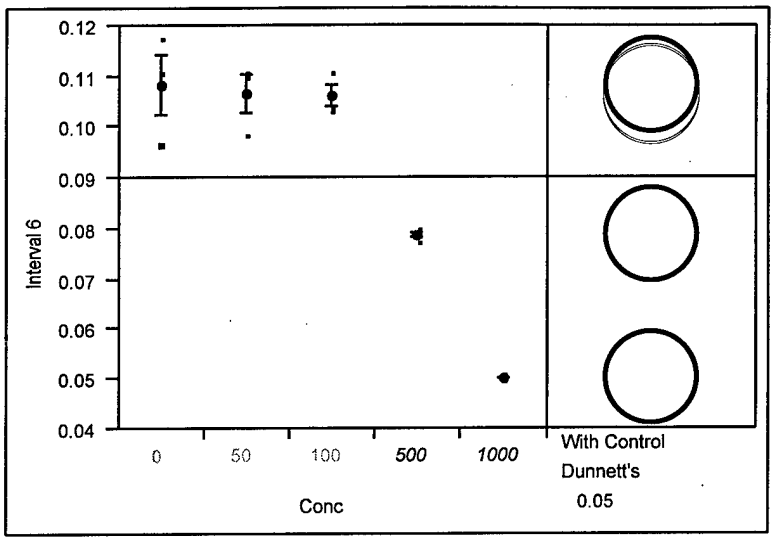


Figure 14. Saturn Interval 6

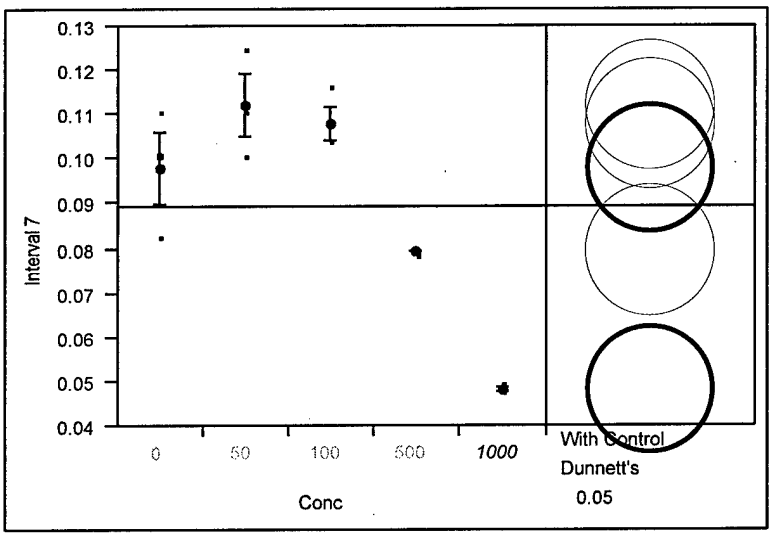


Figure 15. Saturn Interval 7

Table 23. Summary: Saturn Dunnett's Values

Time Interval	Concentration				
	Control (CD_d)	50 ppm	100 ppm	500 ppm	1000 ppm
1	-0.00719[a,b]	-0.00276	0.003172	0.004672	0.023938
2	-0.00877	-0.00767	-0.0076	0.017201	0.036467
3	-0.01077	-0.01077	-0.01037	0.017399	0.039266
4	-0.00823	-0.0056	-0.0045	0.016937	0.040970
5	-0.00946	-0.00686	-0.00573	0.014608	0.043508
6	-0.01421	-0.01234	-0.01194	0.015425	0.043958
7	-0.02117	-0.00707	-0.01117	-0.00231	0.028792

NOTES:
[a]: $|M_{experiment}-M_{control}| - CD_d$
[b]: Negative values indicate no significant difference (at 95% confidence)

Appendix H: *Bacillus* DO Uptake Rate Data

Time Interval (9 min)	Control Microcosm 1 (mg/L-min)	Control Microcosm 2 (mg/L-min)	Control Microcosm 3 (mg/L-min)	Control Mean (mg/L-min)	Control Std Dev	Control Std Error
1	0.0333	0.0356	0.0400	0.0363	0.0034	0.0020
2	0.0411	0.0389	0.0378	0.0393	0.0017	0.0010
3	0.0344	0.0333	0.0278	0.0319	0.0036	0.0021
4	0.0311	0.0367	0.0344	0.0341	0.0028	0.0016
5	0.0322	0.0311	0.0300	0.0311	0.0011	0.0006
6	0.0311	0.0322	0.0278	0.0304	0.0023	0.0013
7	0.0278	0.0289	0.0300	0.0289	0.0011	0.0006

Time Interval (9 min)	50 ppm Microcosm 1 (mg/L-min)	50 ppm Microcosm 2 (mg/L-min)	50 ppm Microcosm 3 (mg/L-min)	50 ppm Mean (mg/L-min)	50 ppm Std Dev	50 ppm Std Error
1	0.0411	0.0400	0.0389	0.0400	0.0011	0.0006
2	0.0344	0.0367	0.0344	0.0352	0.0013	0.0007
3	0.0344	0.0322	0.0289	0.0319	0.0028	0.0016
4	0.0311	0.0400	0.0389	0.0367	0.0048	0.0028
5	0.0322	0.0267	0.0300	0.0296	0.0028	0.0016
6	0.0289	0.0300	0.0256	0.0281	0.0023	0.0013
7	0.0256	0.0311	0.0311	0.0293	0.0032	0.0019

Time Interval (9 min)	100 ppm Microcosm 1 (mg/L-min)	100 ppm Microcosm 2 (mg/L-min)	100 ppm Microcosm 3 (mg/L-min)	100 ppm Mean (mg/L-min)	100 ppm Std Dev	100 ppm Std Error
1	0.0400	0.0422	0.0433	0.0419	0.0017	0.0010
2	0.0356	0.0400	0.0356	0.0370	0.0026	0.0015
3	0.0333	0.0322	0.0333	0.0330	0.0006	0.0004
4	0.0300	0.0378	0.0356	0.0344	0.0040	0.0023
5	0.0322	0.0289	0.0322	0.0311	0.0019	0.0011
6	0.0322	0.0322	0.0289	0.0311	0.0019	0.0011
7	0.0256	0.0267	0.0322	0.0281	0.0036	0.0021

Time Interval (9 min)	500 ppm Microcosm 1 (mg/L-min)	500 ppm Microcosm 2 (mg/L-min)	500 ppm Microcosm 3 (mg/L-min)	500 ppm Mean (mg/L-min)	500 ppm Std Dev	500 ppm Std Error
1	0.0500	0.0422	0.0389	0.0437	0.0057	0.0033
2	0.0411	0.0422	0.0356	0.0396	0.0036	0.0021
3	0.0389	0.0400	0.0378	0.0389	0.0011	0.0006
4	0.0400	0.0400	0.0367	0.0389	0.0019	0.0011
5	0.0378	0.0367	0.0389	0.0378	0.0011	0.0006
6	0.0400	0.0389	0.0400	0.0396	0.0006	0.0004
7	0.0300	0.0322	0.0278	0.0300	0.0022	0.0013

Time Interval (9 min)	1000 ppm Microcosm 1 (mg/L-min)	1000 ppm Microcosm 2 (mg/L-min)	1000 ppm Microcosm 3 (mg/L-min)	1000 ppm Mean (mg/L-min)	1000 ppm Std Dev	1000 ppm Std Error
1	0.0367	0.0333	0.0333	0.0344	0.0019	0.0011
2	0.0311	0.0278	0.0267	0.0285	0.0023	0.0013
3	0.0278	0.0267	0.0222	0.0256	0.0029	0.0017
4	0.0244	0.0267	0.0233	0.0248	0.0017	0.0010
5	0.0289	0.0244	0.0244	0.0259	0.0026	0.0015
6	0.0289	0.0389	0.0333	0.0337	0.0050	0.0029
7	0.0256	0.0133	0.0178	0.0189	0.0062	0.0036

Appendix I: *Bacillus* Dunnett's Comparison Results

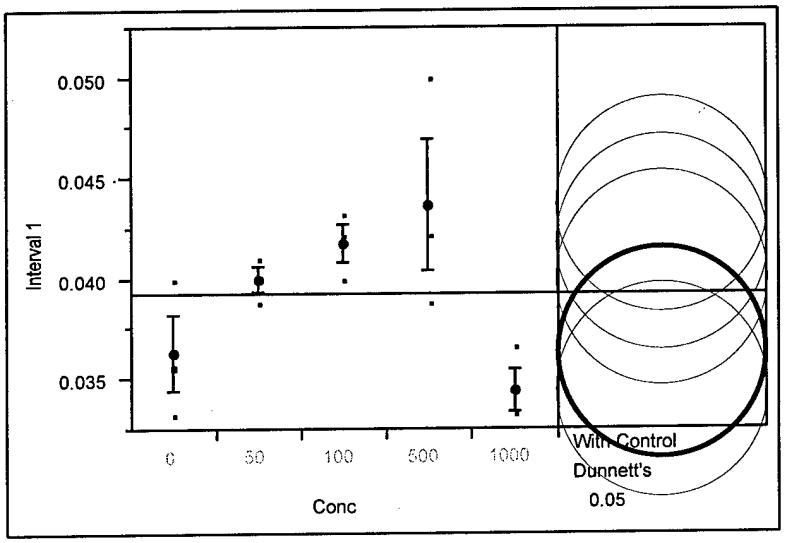


Figure 16. *Bacillus* Interval 1

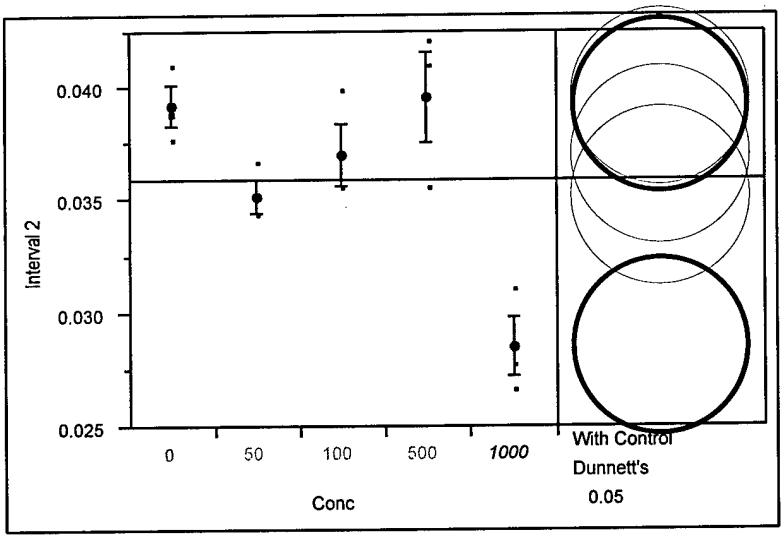


Figure 17. *Bacillus* Interval 2

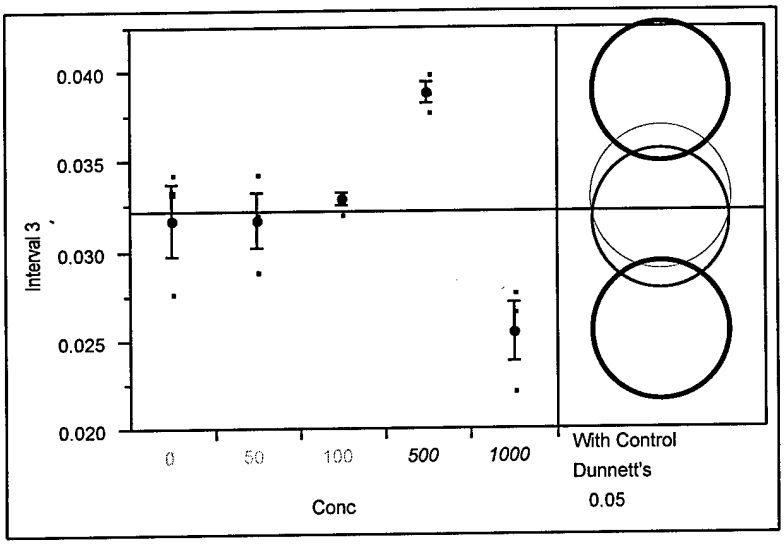


Figure 18. *Bacillus* Interval 3

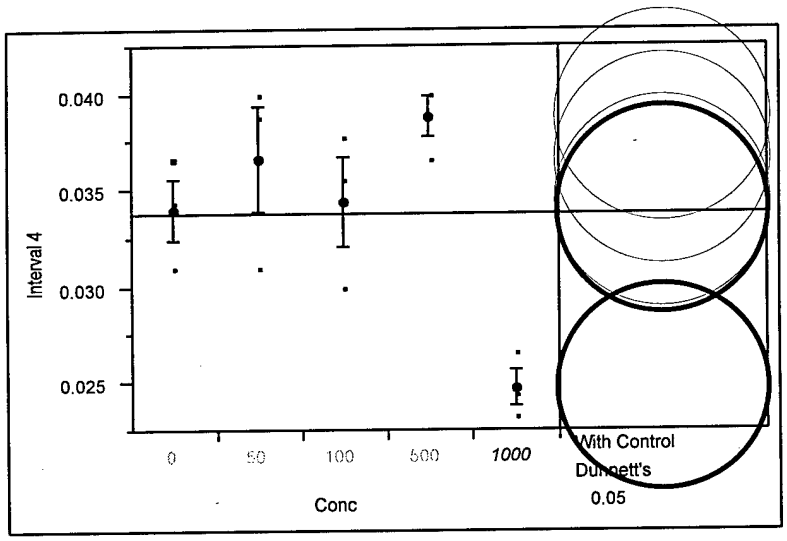


Figure 19. *Bacillus* Interval 4

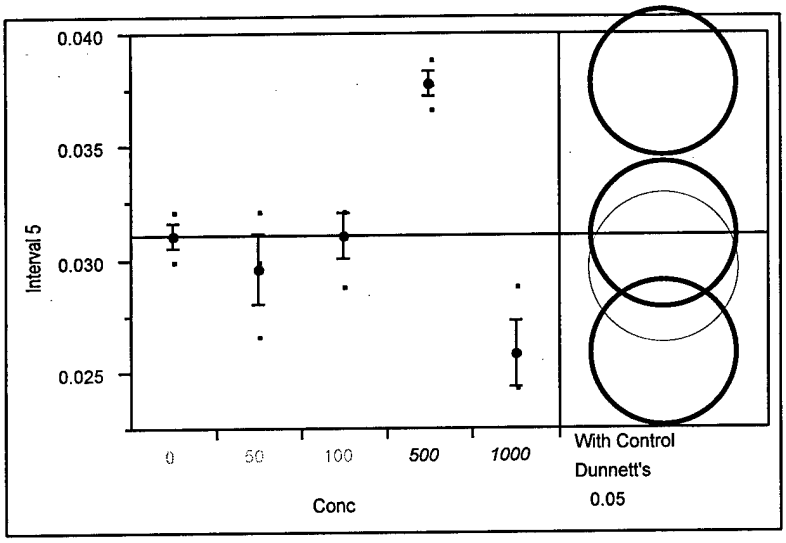


Figure 20. *Bacillus* Interval 5

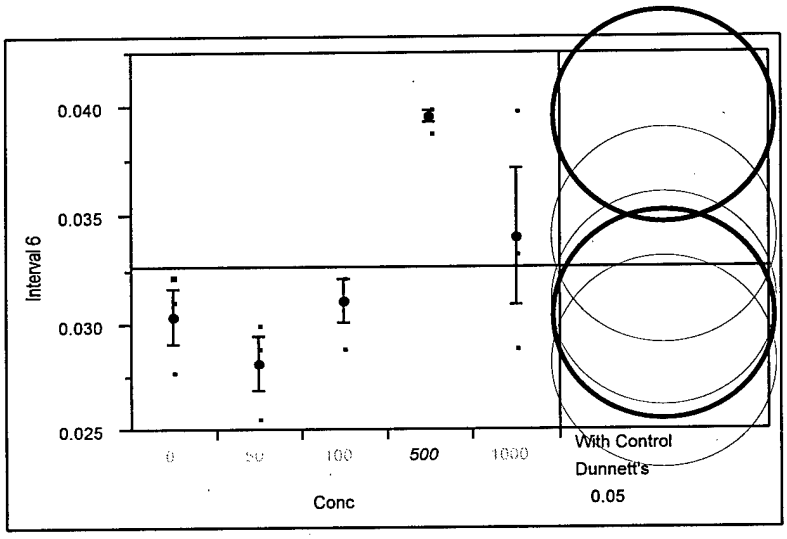


Figure 21. *Bacillus* Interval 6

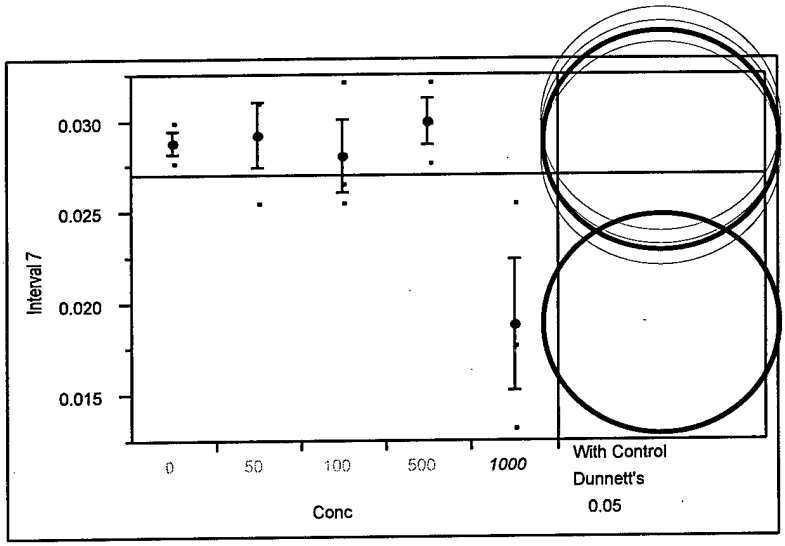


Figure 22. *Bacillus* Interval 7

Table 24. Summary *Bacillus* Dunnett's Values

Time Interval	Concentration				
	Control (CD_d)	50 ppm	100 ppm	500 ppm	1000 ppm
1	-0.00761[a,b]	-0.00391	-0.00208	-0.00021	-0.00574
2	-0.00566	-0.00156	-0.00346	-0.0053	0.005070
3	-0.00584	-0.00584	-0.00474	0.001230	0.000430
4	-0.00778	-0.00518	-0.00738	-0.00294	0.001491
5	-0.00477	-0.00331	-0.00477	0.001925	0.000425
6	-0.00709	-0.00489	-0.00636	0.002173	-0.00343
7	-0.00866	-0.0083	-0.00793	-0.00756	0.001337

NOTES:
[a]: $|M_{experiment}-M_{control}| - CD_d$
[b]: Negative values indicate no significant difference (at 95% confidence)

Appendix J: Mars DO Uptake Rate Data

Time Interval (9 min)	Control Microcosm 1 (mg/L-min)	Control Microcosm 2 (mg/L-min)	Control Microcosm 3 (mg/L-min)	Control Mean (mg/L-min)	Control Std Dev	Control Std Error
1	0.1422	0.1367	0.1456	0.1415	0.0045	0.0026
2	0.1444	0.1422	0.1333	0.1400	0.0059	0.0034
3	0.1344	0.1311	0.1367	0.1341	0.0028	0.0016
4	0.1311	0.1300	0.1300	0.1304	0.0006	0.0004
5	0.1278	0.1244	0.1256	0.1259	0.0017	0.0010
6	0.1133	0.1144	0.1189	0.1156	0.0029	0.0017

Time Interval (9 min)	50 ppm Microcosm 1 (mg/L-min)	50 ppm Microcosm 2 (mg/L-min)	50 ppm Microcosm 3 (mg/L-min)	50 ppm Mean (mg/L-min)	50 ppm Std Dev	50 ppm Std Error
1	0.1344	0.1322	0.1433	0.1367	0.0059	0.0034
2	0.1389	0.1378	0.1322	0.1363	0.0036	0.0021
3	0.1278	0.1278	0.1322	0.1293	0.0026	0.0015
4	0.1267	0.1267	0.1278	0.1270	0.0006	0.0004
5	0.1211	0.1233	0.1256	0.1233	0.0022	0.0013
6	0.1156	0.1156	0.1189	0.1167	0.0019	0.0011

Time Interval (9 min)	100 ppm Microcosm 1 (mg/L-min)	100 ppm Microcosm 2 (mg/L-min)	100 ppm Microcosm 3 (mg/L-min)	100 ppm Mean (mg/L-min)	100 ppm Std Dev	100 ppm Std Error
1	0.1500	0.1433	0.1367	0.1433	0.0067	0.0038
2	0.1444	0.1489	0.1400	0.1444	0.0044	0.0026
3	0.1344	0.1356	0.1333	0.1344	0.0011	0.0006
4	0.1322	0.1367	0.1322	0.1337	0.0026	0.0015
5	0.1267	0.1300	0.1256	0.1274	0.0023	0.0013
6	0.1056	0.1044	0.1156	0.1085	0.0061	0.0035

Time Interval (9 min)	500 ppm Microcosm 1 (mg/L-min)	500 ppm Microcosm 2 (mg/L-min)	500 ppm Microcosm 3 (mg/L-min)	500 ppm Mean (mg/L-min)	500 ppm Std Dev	500 ppm Std Error
1	0.1511	0.1467	0.1456	0.1478	0.0029	0.0017
2	0.1456	0.1433	0.1378	0.1422	0.0040	0.0023
3	0.1400	0.1367	0.1344	0.1370	0.0028	0.0016
4	0.1333	0.1289	0.1289	0.1304	0.0026	0.0015
5	0.1267	0.1200	0.1181	0.1216	0.0045	0.0026
6	0.1144	0.1133	0.1130	0.1136	0.0008	0.0004

Time Interval (9 min)	1000 ppm Microcosm 1 (mg/L-min)	1000 ppm Microcosm 2 (mg/L-min)	1000 ppm Microcosm 3 (mg/L-min)	1000 ppm Mean (mg/L-min)	1000 ppm Std Dev	1000 ppm Std Error
1	0.1467	0.1511	0.1389	0.1456	0.0062	0.0036
2	0.1422	0.1367	0.1311	0.1367	0.0056	0.0032
3	0.1400	0.1356	0.1300	0.1352	0.0050	0.0029
4	0.1300	0.1233	0.1233	0.1256	0.0038	0.0022
5	0.1244	0.1211	0.1167	0.1207	0.0039	0.0023
6	0.1133	0.1144	0.1133	0.1137	0.0006	0.0004

Appendix K: Mars Dunnett's Comparison Results

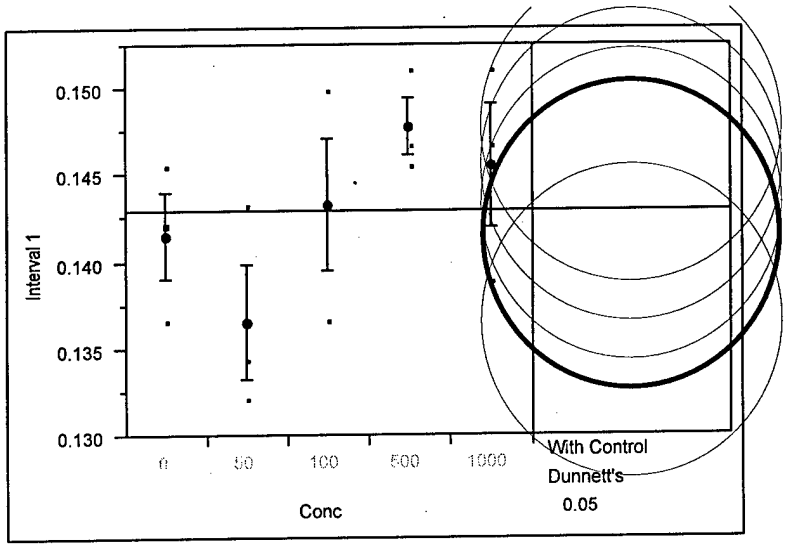


Figure 23. Mars Interval 1

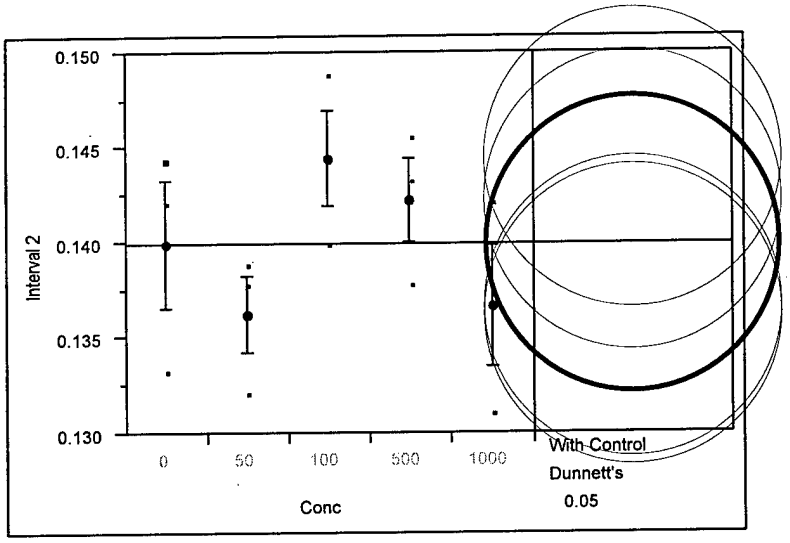


Figure 24. Mars Interval 2

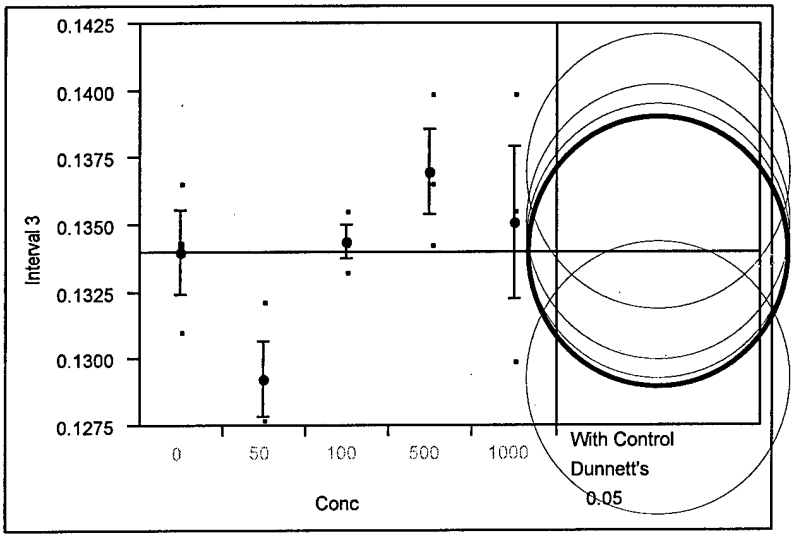


Figure 25. Mars Interval 3

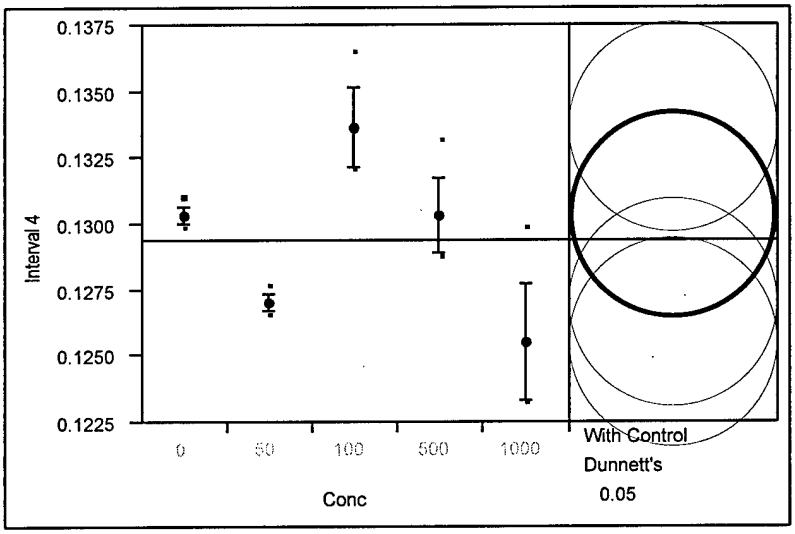


Figure 26. Mars Interval 4

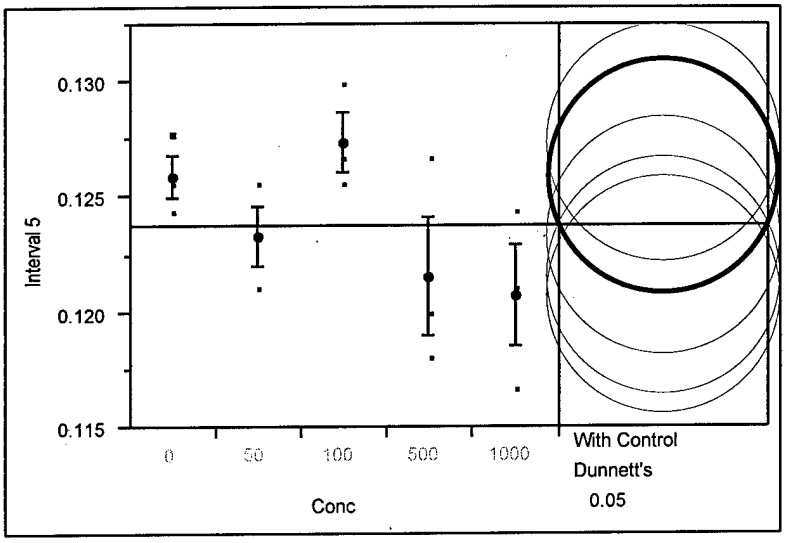


Figure 27. Mars Interval 5

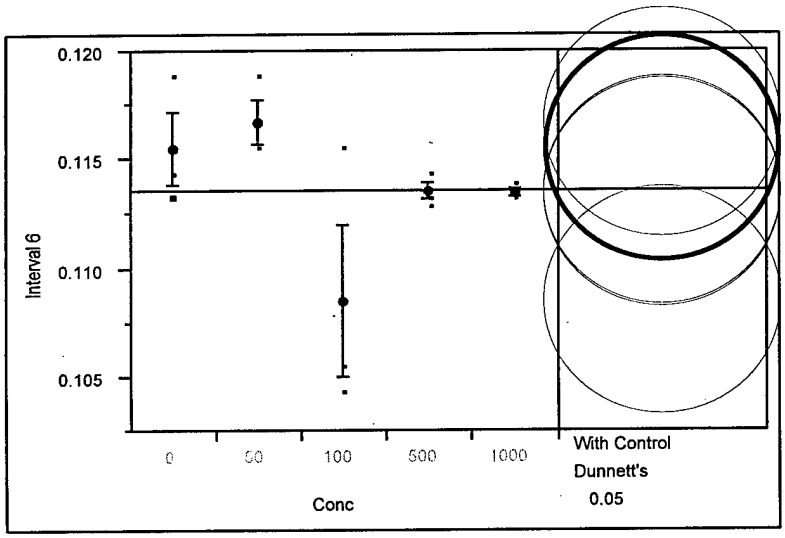


Figure 28. Mars Interval 6

Table 25. Summary Mars Dunnett's Values

Time Interval	Concentration				
	Control (CD_d)	50 ppm	100 ppm	500 ppm	1000 ppm
1	-0.01273[a,b]	-0.00787	-0.0109	-0.00643	-0.00867
2	-0.01128	-0.00761	-0.00681	-0.00901	-0.00798
3	-0.00737	-0.00257	-0.007	-0.0044	-0.00624
4	-0.00568	-0.00238	-0.00235	-0.00568	-0.00085
5	-0.00736	-0.00476	-0.00586	-0.00303	-0.00216
6	-0.00753	-0.00637	-0.00053	-0.00557	-0.00553

NOTES:
[a]: $|M_{experiment}-M_{control}| - CD_d$
[b]: Negative values indicate no significant difference (at 95% confidence)

Appendix L: Microbial Plate Count Data

The number of colonies counted is shown in the table below along with the average, the standard deviation, and the normalized rate.

Table 26. Microbial Plate Count Summary

Microcosm	De I (CFU/uL)	Ctrl (CFU/uL)	50 ppm (CFU/uL)	100 ppm (CFU/uL)	500 ppm (CFU/uL)	1000 ppm (CFU/uL)
1	3	701	690	602	550	385
2	4	806	586	579	661	499
3	2	607	488	682	576	433
Average	3	705	588	621	596	439
Std Dev	1	100	101	54	58	57
Normalized	0.00	1.00	0.83	0.88	0.85	0.62

The Dunnett's comparison graph is shown below.

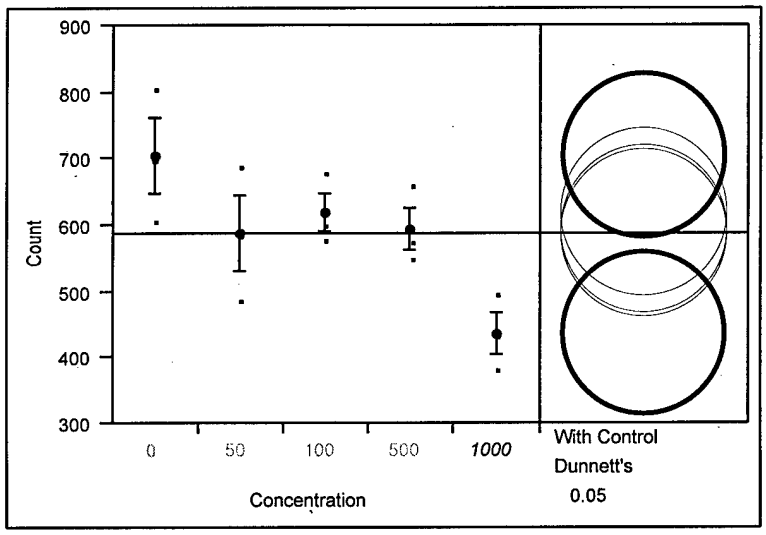


Figure 29. Dunnett's Comparison Colony Count

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Vita

Lieutenant Heather L. Mitchell was born 8 Nov 1974 in Farmington Hills, Michigan. She graduated from Redford Union High School in June 1993. She graduated with a Bachelor of Science in Civil / Environmental Engineering from the United States Air Force Academy in May 1997. She was commissioned on 28 May 1997 with a reserve commission.

She reported to her first duty assignment as part of the 60th Civil Engineer Squadron at Travis AFB, CA in August of 1997. She worked in the Maintenance Engineering element of the operations flight. In August of 1998 she entered the Environmental Engineering and Management program, School of Engineering, Air Force Institute of Technology. Upon graduation she will be assigned to the 51st Civil Engineer Squadron at Osan AB, South Korea.

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